



**US Army Corps
of Engineers**

Engineer Research and
Development Center

Technical Report SERDP-99-4
December 1999

Strategic Environmental Research and Development Program

Explosives Conjugation Products in Remediation Matrices: Final Report

by *Judith C. Pennington, K. A. Thorn, L. S. Inouye,
V. A. McFarland, A. S. Jarvis, C. H. Lutz,
C. A. Hayes, B. E. Porter*

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by Judith C. Pennington, L. S. Inouye,
V. A. McFarland, A. S. Jarvis, C. H. Lutz

Environmental Laboratory
U.S. Army Engineer Research and Development Center
3909 Halls Ferry Road
Vicksburg, MS 39180-6199

K. A. Thorn

National Water Quality Laboratory
U.S. Geological Survey
P.O. Box 25046
Denver, CO 80225-0046

C. A. Hayes, B. E. Porter

ASCI Corporation
1365 Beverly Road
McLean, VA 22101

Final report

Approved for public release; distribution is unlimited

Army Engineer Research and Development Center Cataloging-in-Publication Data

Explosives conjugation products in remediation matrices : final report / by Judith C. Pennington... [et al.] ; prepared for U.S. Army Corps of Engineers.

50 p. : ill. ; 28 cm.—(Technical report ; SERDP-99-4)

Includes bibliographic references.

1. Soil remediation. 2. Nitrotoluene. 3. Humic acid. I. Pennington, Judith C. II. United States. Army. Corps of Engineers. III. U.S. Army Engineer Research and Development Center. IV. Strategic Environmental Research and Development Program (U.S.) V. Series: Technical report SERDP ; 99-4. TA7 E8 no.SERDP-99-4

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Preface

This report was prepared by the U.S. Army Engineer Research and Development Center (ERDC) Environmental Laboratory (EL) in association with the U.S. Geological Survey (USGS), Denver, CO, and AScI Corporation, McLean, VA. The research was sponsored by the Strategic Environmental Research and Development Program (SERDP), Arlington, VA, Dr. John Harrison (retired), EL, and Mr. Bradley P. Smith, Arlington, VA, Executive Directors, Project CU715. Dr. M. John Cullinane was the ERDC Program Manager for the SERDP. The Principal Investigator was Dr. Judith C. Pennington, EL.

The authors of this report were as follows:

Chapter 1	J. C. Pennington, EL
Chapter 2	K. A. Thorn, USGS; J. C. Pennington, EL; C. A. Hayes and B. E. Porter, AScI
Chapter 3	L. S. Inouye, V. A. McFarland, A. S. Jarvis, and C. H. Lutz, EL
Chapter 4	J. C. Pennington, EL

Technical assistance with laboratory reactions and nuclear magnetic resonance analyses was provided by Ms. Kay Kennedy, USGS. This report was reviewed by Dr. James M. Brannon, EL. The study was conducted under the general supervision of Dr. Richard E. Price, Chief, Environmental Processes and Effects Division, and Dr. John W. Keeley, Acting Director, EL.

At the time of publication of this report, Dr. Lewis E. Link was Acting Director of ERDC, and COL Robin R. Cababa, EN, was Commander.

This report should be cited as follows:

Pennington, J. C., Thorn, K. A., Inouye, L. S., McFarland, V. A., Jarvis, A. S., Lutz, C. H., Hayes, C. A., and Porter, B. E. (1999). "Explosives conjugation products in remediation matrices: Final report," Technical Report SERDP-99-4, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

1 Introduction

Background

Research from several sources indicates that the explosive 2,4,6-trinitrotoluene (TNT) becomes immobilized in soil and compost systems. Some of the earliest evidence for immobilization of TNT in soils came when radiolabeled TNT and 4-amino-2,6-dinitrotoluene (4ADNT), a transformation product of TNT, were added to soils for a plant uptake study (Pennington 1988). Comparisons of percent recoveries of ^{14}C by solvent (acetone) extraction and from complete combustion of soil revealed that an average of four times as much of the added radioactivity was recovered by combustion. Solvent extraction failed to remove the immobilized residues of TNT or of 4ADNT. These results indicated that standard analytical techniques, which require solvent extraction of the TNT and its transformation products, fail to reveal a significant quantity of immobilized contaminant.

Evidence for immobilization of TNT in compost resulted from a study in which radiolabeled TNT was added to soil prior to composting (Pennington et al. 1995). After 20 days, the compost was fractionated into solvent-extractable (ether followed by acetonitrile), cellulose, fulvic acid, humic acid, and humin components. Less than 20 percent of the added radioactivity was solvent extractable. Since TNT is much less soluble in water than in these organic solvents, this result suggested that mobilization of TNT or of its products from compost by water in the environment is limited. More than 30 percent of the added radioactivity was associated with the cellulose fraction, and more than 20 percent was associated with the humin. Cellulose is a biodegradable component of organic matter that may release the contaminant in some form, but humin is extremely recalcitrant to further degradation in the environment.

Results of these studies indicated that immobilization of TNT and/or its transformation products is a significant fate process. An understanding of the nature and mechanisms of immobilization may be very important to the development of effective treatment technologies. Furthermore, if the extent of immobilization in the environment approaches the extent observed in the laboratory, immobilization may exceed adsorption and degradation processes in importance as a contaminant fate process. The information generated by this research may be used to develop natural attenuation of explosives in soils and groundwater, to assess the ultimate

fate of explosives in compost, and to estimate the bioavailability of explosives in biotreatment systems.

Two interim reports describing results of research prior to Fiscal Year 1999 (FY99) (Pennington et al. 1997, 1998) included the results of the following research areas:

Toxicology: Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene(TNT) and its metabolites

Dialysis Partitioning: Trinitrotoluene and metabolites partitioning to humic acid

Hydrolytic Release: Hydrolytic release of bound residues from composted TNT-contaminated soil

Microbial Degradation: Microbial degradation of conjugated fractions

Surface Plasmon Resonance: Analysis of coupling affinities among TNT degradation products and humic acid based on surface plasmon resonance

Covalent Binding: Use of ^{15}N NMR to evaluate covalent bonding of TNT transformation products to surrogates for functional groups on soil humic acid molecules

Two of the research areas, toxicology and covalent binding, continued in FY99 and are the subject of this final project report. The following is a brief summary of results presented in the FY97 and FY98 interim reports.

Toxicology

This study was undertaken to examine the *in vitro* cytotoxicity and mutagenicity of TNT and several of its reduction products. Cytotoxicity assays were conducted using two cultured cell lines, Reuber H35 H4IIE rat hepatoma cells and Chinese Hamster Ovary-K1(CHO) cells. Mutagenicity of the compounds was assessed using Ames and Mutatox assays. Results indicated that TNT, 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), 4,4', 6,6'-tetranitro-2,2'-azoxytoluene (2,2'AZOXY), and 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'AZOXY) were equally cytotoxic to H4IIE and CHO cells. Two other TNT transformation products, 4ADNT and 2-amino-4,6-dinitrotoluene (2ADNT), were cytotoxic to H4IIE cells, but not to CHO cells. Another transformation product, 2,4-diamino-6-nitrotoluene (2,4DANT), was noncytotoxic to either cell type. These results indicated that the transformation products of TNT mentioned above are as cytotoxic as, or only moderately less cytotoxic than, TNT *in vitro*. Results of Ames assays indicated that TNT with and without metabolic activation was slightly mutagenic to strain TA98, but not mutagenic to strain TA100. Only 2ADNT was slightly mutagenic to strain TA98 without activation. Both 2ADNT and 2,2'AZOXY exhibited mutagenicity to strain TA100 with and without activation, while 4ADNT was slightly mutagenic without activation. In Mutatox assays, TNT was mutagenic without S9 activation, while 2ADNT, 4ADNT, and 2,4DANT were mutagenic both with and without S9 activation.

One significant generalization of these results is that while the two most commonly detected transformation products of TNT, 2ADNT and 4ADNT, are equal to or only slightly less toxic than the parent compound, their mutagenicity as measured by the Mutatox assay is significantly higher than that of TNT (Honeycutt, Jarvis, and McFarland 1996; Pennington et al. 1997).

Results of Mutatox and Ames assays of TNT and the amino transformation products indicated none to moderate microbial genotoxicity. In general, the monoamino transformation products of TNT were equally or only slightly less toxic than the parent compound. However, toxicity of the compounds is best assessed in tests that take bioavailability into account. Therefore, a battery of earthworm bioassays on TNT-contaminated compost and soils were conducted. The battery included acute toxicity, chronic toxicity, avoidance behavior, metabolism and oxidative stress, immunotoxicity, and genotoxicity. The 14-day LC_{50} of TNT in soil was 353 mg kg^{-1} . Attempts to define similar toxicity for RDX and HMX were abandoned at soil concentrations of $4,000 \text{ mg kg}^{-1}$ when no earthworms died. Although HMX and RDX in soil were not acutely toxic to earthworms, their negative effect on reproduction at 400 mg kg^{-1} was severe. Earthworms exhibited high chronic toxicity to finished TNT compost; only 35 percent survival after 8 days compared with 96 percent for controls after 21 days of exposure. This toxicity was suspected to be due to the 4ADNT rather than to TNT because no TNT was extractable from the matrix while 4ADNT was found at 4.55 mg kg^{-1} . Earthworms avoided the finished compost, suggesting that colonization of finished compost by earthworms is not likely. The residual TNT transformation products may induce stress in the worms causing avoidance. Oxidative stress effects on several enzymes and protein were observed in worms exposed to TNT-contaminated soils and compost. TNT-contaminated soils reduced immunocompetence in the worms, but was not genotoxic as measured by DNA single-strand breakage. However, finished compost was highly genotoxic. This result was attributed to the presence of extractable transformation products of TNT (Jarvis, McFarland, and Honeycutt 1998; Pennington et al. 1998).

Dialysis partitioning

These studies were conducted to quantify the adsorption of TNT and several of its transformation products to humic acid. Equilibrium dialysis was used to determine the effects of contaminant concentration, humic acid concentration, pH, and ionic strength on the formation kinetics and on extent of interaction. Results indicated that TNT, 2,6DANT, and 2ADNT bind to humic acid slowly. As the concentration of humic acid increased, the extent of binding decreased. As pH increased, the extent of binding increased. Nearly a twofold increase in binding of TNT was observed for a fivefold increase in ionic strength. A linear model best fit the 2,6DANT isotherm data, while a Langmuir model best fit the TNT data. The maximum binding density of TNT for humic acid was 6 to $30 \text{ } \mu\text{M}$ TNT per μM humic acid (Li et al. 1997; Pennington et al. 1997).

Hydrolytic release

The objectives of this study were to differentiate between bound and unbound explosives and their transformation products in composted soil, to determine the time course of metabolite evolution and binding during composting, and to evaluate various hydrolysis methods for assaying bound products. Compost samples were subjected to two hydrolysis procedures: acid (50 percent aqueous sulfuric acid) and base (0.5 M sodium hydroxide) followed by acid (concentrated sulfuric acid). Results indicated that significant quantities of identifiable amino-dinitrotoluenes and diaminonitrotoluenes were released after solvent-extracted residues were subjected to acid or base hydrolysis. However, no TNT was released. The concentration of hydrolyzable metabolites reached a maximum at 10 days of composting, then decreased through 40 days. This study produced a method for removing and identifying a significant portion of the immobilized products of TNT (Thorne and Leggett 1997; Pennington et al. 1997).

The hydrolysis regime developed in this study is effective in removing the weakly conjugated amines that are subject to further reaction. This pool constitutes the potentially available products resulting from treatment at the stage where the analysis is conducted. When treatment takes the reaction sequence to completion, no hydrolyzable products are released. The hydrolysis method can, therefore, serve as an indicator of treatment progression and/or effectiveness. The method is not sufficiently sensitive for application to immobilization processes in soils because of the relatively small amount of organic matter in soils (Thorne and Leggett 1999; Pennington et al. 1998).

Microbial Degradation

An important question concerning the remediation technologies in which immobilization products are formed is long-term stability of the product. To address this concern, the biodegradability of immobilized products was examined. This study had two objectives. The first objective was to determine the ability of several common enzymes to modify TNT and/or mediate reactions between TNT and major soil organic components. These enzymes are typically active in degradation of aromatic contaminants and are common in microorganisms. The second objective was to evaluate the ability of soil microflora to mineralize radiolabeled TNT that has been immobilized in soil and/or compost. Results on the first objective showed that none of the three enzymes, polyphenol oxidase, laccase, or polyphenol oxidase, exhibited activity against TNT. Results for the second objective showed active enrichment cultures on fractions of compost: acetonitrile extract, cellulose, fulvic acid, humic acid, and humin. Enrichment cultures growing on fractions from radiolabeled compost exhibited mineralization ($^{14}\text{CO}_2$) from cellulose and fulvic acid fractions only (Pennington et al. 1997).

Explosives residuals in composted TNT-contaminated soils were not degradable by microorganisms. Very few microorganisms were able to grow on the compost because it failed to provide sufficient carbon and energy. When the

most degradable organic fraction, cellulose, was subjected to degradation studies independently of the rest of the compost matrix, microorganisms were able to mineralize significant quantities of added radiolabeled TNT (up to 23 percent). Moderate mineralization activity was also observed in the fulvic acid fraction. These results demonstrate the limits of degradation potential in finished compost. Since the fractions are dispersed in the compost matrix, their degradation potential in whole compost is extremely small. Therefore, significant release of environmentally hazardous products from finished compost by microbial action is unlikely (Pennington et al. 1998).

Surface plasmon resonance

The Biospecific Interaction Analysis System presents a method by which coupling affinity between molecules can be assessed. This instrumental system is extremely sensitive for investigating molecular interactions at the surface of a sensor chip using *surface plasmon resonance* to analyze the concentration of the biomolecules attached to the chip surface. The rationale was to bind the smaller molecules of TNT (or transformation products) to the surface matrix of the chip and introduce the larger humic acid molecules over the surface. If the humic acid interacted with the TNT, the kinetics could be followed by minute changes in weight to which the instrument is extremely sensitive. Since this is a state-of-the-art technology requiring adaptation to procedures of this study, great care was taken to validate performance and execution of tests. Results indicated that neither TNT nor two of its transformation products, 4ADNT and 2,6DANT, could be immobilized on the chip surface. Various modifications of pH, concentration, and flow rate were tried without success. Binding of the humic acid, rather than the explosive, was also explored without success. Apparently, binding of a substrate to the chip material is chemical class specific. Protein experiments confirmed that the system functions well with "biospecific" compounds, but not with all other classes of organic compounds (Pennington et al. 1998).

Covalent binding

Recent research using liquid- and solid-state ^{15}N -nuclear magnetic resonance (NMR) has demonstrated that aniline binds covalently to isolated humic substances and to the organic matter in whole soils and peat (Thorn et al. 1996a,b). Aniline reacts with isolated fulvic and humic acid in the presence and absence of phenoxidase enzymes or metal catalysts. In the absence of catalysts, aniline undergoes a complex series of nucleophilic addition reactions with quinones and other carbonyl groups to form both heterocyclic and nonheterocyclic condensation products. In the presence of the enzyme or metal catalyst, aniline undergoes free radical coupling reactions together with nucleophilic addition reactions with the humic substances. Since common reductive products of TNT, i.e., 2ADNT, 4ADNT, 2,4DANT, 2,6DANT, chemically resemble aniline in functionality groups, the premise that these reductive products may mimic the coupling reactions of aniline with humic acid was likely.

The objectives of this study were (a) to demonstrate covalent binding of 4ADNT, 2ADNT, 2,6DANT, and 2,4DANT with model carbonyl compounds, with humic substances, and with whole compost and soil and (b) to determine the chemical lability of the bound amino residues. The amines were reacted with model carbonyl compounds (e.g., 4-methylcatechol, 1,4-benzoquinone, and glucose). The ^{15}N NMR chemical shifts of the condensation products were measured using liquid-state ^{15}N NMR. The spectra of product mixtures from amine reactions were also measured. Reference fulvic and humic acid (from the International Humic Substances Society) were reacted with TNT reduction products with and without catalysts (liquid- and solid-phase NMR). Organic matter fractions of compost were extracted and reacted with TNT reduction products. The products were analyzed by liquid- and/or solid-state ^{15}N -NMR. Time-series interactions ranging from weeks to months were conducted to determine interactions between reduction products and both compost and surface soils. To determine the chemical lability of bound residues, exchange of the bound residues with other amines or release of the residues by hydrolytic enzymes and microbial degradation were tried. Results provided a chemical definition of a significant immobilization process for TNT and TNT transformation products (Thorn 1997; Pennington et al. 1998).

The monoamino and diamino transformation products of TNT covalently bind to carbonyl compounds, humic substances, and whole peat in compost and soil. The number of potential products forming from the interaction of the diamino transformation products of TNT with humic functional groups is greater than the number from the monoamino transformation products. This is attributed to the higher pKa and greater nucleophilicity of the diamino products. Furthermore, the 4ADNT produces more products than the 2ADNT. This is due to steric hindrance effects in the 2ADNT.

The enzyme peroxidase failed to catalyze auto condensation of the monoamines. However, the enzyme catalyzed the formation of several products from 2,4DANT, some identified (e.g., azoxybenzenes, imines, hydrazines) and some uncertain. Fewer products were observed from 2,6DNT. Polymerization of conniferyl alcohol by peroxidase is a potential biosynthetic pathway for the formation of lignin in woody plants. Peroxidase catalyzed reactions of the monoamines and diamines with conniferyl alcohol resulting in complex products. These results demonstrate the possibility of such reactions in plants.

Reactions between the diamines and humic acid in the presence of the enzyme resulted in greater imine formation than heterocyclic nitrogen formation. Deamination with release of ammonia was also observed. In the absence of the enzyme, the relative proportion of imines and ammonia was less. Reactions between the monoamines and humic acid result in more heterocyclic products than imines. The enzyme also enhanced these reactions.

Spectral analyses before and after hydrolysis of TNT that had been reacted with peat indicated that some bonds were not hydrolyzable. Aminoquinone,

amide, and imine linkages were hydrolyzable, while aminohydroquinone bonds persisted. Heterocyclic products were resistant to hydrolysis.

The products of the interactions between TNT transformation products and humic substances are complex. The progression of covalent bonding reactions results in products exhibiting various potentials for subsequent release. However, a significant proportion of these reactions are extremely stable. Therefore, long-term stability of the products of these reactions, and, consequently, of the effectiveness of the remediation processes in which they occur, is a function of the local environment, the transformation products available for reaction, and the progression of the reactions (Pennington et al. 1998).

Objectives

The broad objectives of this study were to determine the nature and mechanism of the immobilization interactions between TNT and soil components, the long-term stability, biodegradability, and toxicity of the products of those interactions, and methods for detecting products of the interactions. Specific objectives of each study are presented within each chapter. Only the toxicology and covalent binding studies continued in FY99 and are reported here.

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2 Reduction and Subsequent Bonding of TNT in Compost

Introduction

The purpose of these studies was to determine the incidence in actual compost of covalently bound products that had been observed with surrogates of humic acid functional groups. A previous report established that the major reductive degradation products of TNT, 4ADNT, 2ADNT, 2,4DANT, and 2,6DANT, undergo covalent binding with soil humic and fulvic acid, model lignin and quinone compounds, and the organic matter of whole peat (Thorn 1998). The monoamines, 4ADNT and 2ADNT, and the diamines, 2,4DANT and 2,6DANT, were synthesized with ^{15}N labels in the amine positions. The amines were reacted with the model compounds and humic materials in the presence and absence of horseradish peroxidase enzyme as catalyst. Liquid- and solid-state ^{15}N NMR analyses confirmed that the labeled amino groups underwent nucleophilic addition reactions with quinone and other carbonyl groups to form both heterocyclic and nonheterocyclic condensation products. The monoamines were observed to be less reactive than the diamines. Differences in the distribution and quantities of the condensation products were observed between the peroxidase catalyzed and noncatalyzed reactions. The first part of this chapter describes preparation and analysis of the compost, while the remainder of the chapter describes the NMR analyses.

Part I: Compost Preparation

Objectives and approach

The objective of Part I was to produce compost samples containing immobilized products of TNT enriched in ^{15}N to enhance NMR analysis. A preliminary experiment was conducted to determine the capacity of the compost matrix for TNT so that the maximum amount of heavy isotope could be immobilized with

minimal unbound excess. Another preliminary experiment was conducted with [^{14}C]TNT to ensure quantitative determinations of bound fractions and mass balance. The compost was fractionated to measure the residues in the acetonitrile extractable, cellulose, humic acid, fulvic acid, and humin fractions. Results of this preliminary experiment were used to optimize generation of compost enriched with [^{15}N]-labeled immobilization products. Finally, [^{15}N]TNT compost was generated for NMR analysis.

Material and methods

Soil preparation. Grange Hall soil collected near Vicksburg, MS, was prepared by air-drying and sieving (Number 10 sieve, 1.65 mm) to remove any coarse gravel (Table 1). Soil for the preliminary experiment was amended with 317 μg [^{14}C]TNT¹ (New England Nuclear, Boston, MA) per gram of soil on an oven-dried weight basis (ODW). The [^{14}C]TNT was added as a methanol solution to 1:4 soil to water slurries. The remaining TNT concentration was achieved by adding appropriate amounts of unlabeled crystalline TNT to the slurries. Soil for the ^{15}N enriched compost was amended with 200 mg [^{15}N]TNT per gram of soil. Slurries were placed on a rotatory shaker (New Brunswick Scientific, New Brunswick, NJ) at approximately 400 rotations per minute over night in a cold room (4 °C). Slurries were added to other compost amendments and comprised 10 percent of the compost wet weight.

Table 1 Characteristics of Grange Hall Soil	
Soil Property	Value
Classification (Natural Resources Conservation Service soil survey map)	Tunica; clayey over loamy, montmorillonitic, nonacid, thermic Vertic Haplaquept
pH	8.2
Organic carbon, %	0.29
Cation exchange capacity, meq/100 g	16.7
Sand, %	39
Silt, %	51
Clay, %	10

Compost preparation. The compost mixture consisted of 33 percent green cow manure, 22 percent alfalfa, 6 percent chopped apples, 22 percent sawdust, and 17 percent chopped potatoes. All components were added as percent of wet

¹ Specific activity of 26.3 mCi/mmol; radiological purity >99 percent by thin-layer chromatography; chemical purity >99 percent by high-performance liquid chromatography with an ultraviolet detector.

weight. All amendments were of particle size 10 mm or less. The finer particles were added first, with successively larger particle-sized amendments added as mixing continued. The final mixture was moistened to 75 percent water by weight. Compost for each reactor was prepared separately.

Reactors. The test chambers consisted of wide-mouthed glass canning jars (473 cm³) with modified lids. The chambers were incubated in a water bath maintained at 55 °C. Air flow was from the bottom at 10 ml/min. Temperature was monitored automatically by thermocouples in the center of the compost. A complete description of the configuration and operation of the chambers is given elsewhere (Pennington et al. 1995). Compost was incubated for 20 days.

Analysis of compost. Compost from the preliminary experiment was analyzed before and after incubation by complete combustion in a Model 307 Packard Sample Oxidizer (Packard Instruments, Meridan, CT). Oxidized carbon was trapped as CO₂ in Carbo-Sorb and Permafluor Liquid Scintillation Cocktail (Packard Instruments, Meridan, CT) and assayed by liquid scintillation counting on a Packard Tricarb 2500TR Liquid Scintillation Counter (Packard Instruments, Meridan, CT). The finished compost was fractionated into solvent extractable, cellulose, humic acid, fulvic acid, and humin according to procedures described in Pennington et al. (1995). Fractions were assayed for radioactivity. The finished compost was also extracted overnight in methanol with sonication. The extracts were analyzed by high-performance liquid chromatography (HPLC) according to U.S. Environmental Protection Agency (EPA) SW-846 Method 8330 (EPA 1994). Analytes assayed included TNT, 1,3,5-trinitrobenzene (TNB), 1,3-dinitrobenzene (DNB), 4ADNT, 2ADNT, 2,6-dinitrotoluene (2,6DNT), 2,4-dinitrotoluene (2,4DNT), nitrobenzene (NB), 2-nitrotoluene (2NT), 3-nitrotoluene (3NT), 2,6-diamino-4-nitrotoluene (2,6DANT), 2,4DANT, and 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'AZOXY). The ¹⁵N enriched compost was analyzed by HPLC prior to NMR analyses (see Part II).

Capacity of compost for immobilization. This preliminary experiment was conducted to determine the capacity of the compost matrix for TNT so that the maximum amount of heavy isotope could be immobilized with minimal unbound excess. Soil was amended with three concentrations of TNT (2, 20, and 200 mg per gram of soil ODW) to achieve a final concentration of 200, 2,000, and 20,000 µg TNT per gram of compost as described in previous "Soil preparation" paragraph. Reactors were prepared in duplicate with one reactor left unamended to serve as a control. Two reactors received 2,000 mg of TNT into the compost mix without soil to observe soil effects on immobilization.

Bound fractions and mass balance. Soils for six reactors were amended at 20,000 µg TNT per gram of compost by adding the appropriate quantity of TNT to a 1:4 soil:water slurry and shaking on a rotating shaker over night at 4 °C. The slurry for each reactor was prepared separately and added to the compost mixture for individual compost reactors. Each soil batch also received 300 µg of [¹⁴C]TNT per gram of soil (ODW). Two reactors were unamended to serve as controls. Compost reactors were incubated as described above. Radiological

mass balance was determined by complete combustion analysis of T_0 and T_{20} compost subsamples. Subsamples from each reactor were also analyzed by HPLC as described above. The remainder of the six T_{20} compost treatment replicates were combined as were the remainder of the two compost control replicates, and each was subjected to fractionation according to procedures described by Pennington et al. (1995). Each fraction was subsequently assayed by liquid scintillation counting.

Preparation of [^{14}N]TNT-labeled compost. Two reactors were prepared from soils amended with 200 mg [^{15}N]TNT per gram of soil. To provide total mass data, four reactors were prepared with 1,519 μg [^{14}C]TNT and sufficient unlabeled TNT to yield a total of 200 mg TNT per gram of soil. One reactor was prepared with 200 mg unlabeled TNT per gram of soil. One reactor was an untreated control that served as background. Composting was conducted as described above. Twenty-day radiolabeled samples were analyzed for radioactivity by complete combustion and liquid scintillation counting; all twenty-day samples were analyzed for TNT and TNT transformation products by HPLC; twenty-day ^{15}N -labeled samples were analyzed by NMR (see Part II).

Part II: NMR Analyses

Objectives and approach

In the present set of experiments, in which TNT labeled with ^{15}N in the nitro groups was added to the Grange Hall soil and composted, the objective was to observe in situ both the reduction of the labeled nitro groups to amines and their subsequent condensation with organic matter in the compost and confirm whether the same types of bonds formed between the individual amines and humic acid are reproduced in the compost. In contrast to laboratory experiments in which the individual amines are reacted with humic substances or model compounds under controlled conditions, the composting environment encompasses a potentially more complicated set of reactions. For example, the transitory nitroso and hydroxylamino intermediates formed as the TNT is reduced to the amines are potentially reactive. The nitroso group can act as an electrophile and the hydroxylamino group as a nucleophile. Nitroso groups may be subject to nucleophilic attack by naturally occurring amines and thiols (March 1992).

Materials and methods

Compost samples. Three compost samples, each comprising the contents of a single reactor, were received from the U.S. Army Engineer Research and Development Center (ERDC), refrigerated, and undried. Two of the three samples (2KT N15 and 3KT N15) represented soil samples composted with the ^{15}N -labeled TNT (2,4,6-trinitrotoluene- $^{15}\text{N}_3$). The third sample (1KTblank) represented a blank compost (no TNT added). Samples 2KTN15 and 1KTblank were dried in a

desiccator and then ground in a mortar and pestle to achieve as homogeneous a particle size as possible without performing an actual physical size or density fractionation. Solid-state ^{15}N and ^{13}C NMR spectra were recorded on the samples. Sample 2KTN15 was then extracted with 100 percent acetonitrile. Approximately 0.360 g of the compost was sonicated in 100-ml acetonitrile for 8 hr and then filtered through a 40- to 60- μm fritted glass filter. The compost was air-dried and desiccated and the solid-state ^{15}N and ^{13}C spectra re-recorded.

NMR spectrometry. Solid-state CP/MAS (cross polarization/magic angle spinning) ^{15}N NMR was recorded on a Chemagnetics CMX-200 NMR spectrometer at a nitrogen resonant frequency of 20.3 MHz, using a 7.5-mm ceramic probe (zirconium pencil rotors). Acquisition parameters included a 30,000-Hz spectral window, 17.051-ms acquisition time, 2.0- or 5.0-ms contact time, 0.5-s pulse delay, and spinning rate of 5,000 Hz. Chemical shifts were referenced to glycine, taken as 32.6 ppm. Solid-state CP/MAS ^{13}C NMR spectra of the compost samples were acquired using a 30,000-Hz spectral window, 17.051-ms acquisition time, 2.0-ms contact time, 1.0-s pulse delay, and spinning rate of 5,000 Hz. Carbon-13 chemical shifts were referenced to hexamethylbenzene.

Results

Part I: Compost preparation

Capacity of compost for immobilization. Results of the preliminary experiment indicated that a significant mass of TNT (18,430 μg per gram of compost, or 92 percent of added TNT) was immobilized at the upper limit of the practical quantity of $[^{15}\text{N}]\text{TNT}$ available (Table 2). Therefore, the $[^{15}\text{N}]\text{TNT}$ compost was amended at 20,000 $\mu\text{g/g}$. The reactors receiving a direct amendment of TNT without amended soil exhibited significantly less immobilization. This result suggests a significant role for the soil in immobilization processes.

Table 2 Quantity of TNT Immobilized after 20 Days of Composting			
Initial Concentration $\mu\text{g/g}$	T_{20} Concentration $\mu\text{g/g}^1$	Immobilized	
		Percent	Mass, g/g
200	1.26 ± 1.63	99	199
2,000	1.60 ± 0.47	99	1,998
20,000	$1,570 \pm 438$	92	18,430
40,000 (no soil)	$24,400 \pm 2,546$	39	15,600
Control (no TNT)	<0.100	na ²	na
¹ Values represent means of two replicate compost reactors \pm standard deviation.			
² na indicates not applicable.			

Bound fractions and mass balance. Total recoveries by complete combustion analysis of T₀ and T₂₀ compost were good (Table 3). Recoveries at T₀ did not differ significantly from recoveries at T₂₀.

Table 3 Recoveries of Radioactivity from [¹⁴C]TNT Composted Soil		
	T ₀	T ₂₀
Treatments ¹	84 ± 6.2	82 ± 7.4
Controls ²	<0.20	<0.20
¹ Values represent the mean of six replicates ± standard deviation		
² Values represent the mean of two replicates ± standard deviation		

Recoveries of radioactivity from the various compost fractions were lower than but proportional to those observed by Pennington et al. (1995) (Table 4).

Table 4 Recoveries of Radioactivity and Equivalent TNT Mass¹ from [¹⁴C]TNT Composted Soil after Fractionation of 20-Day Samples		
Fraction	Recovery, %	Equivalent TNT mass, µg/g
Acetonitrile	4.21	8,420
Cellulose	56.24	112,480
Humic Acid	0.54	1,080
Fulvic Acid	1.46	2,920
Humin	6.42	12,840
Total	69.50	139,000
¹ Assumes that all radioactivity recovered is TNT.		

Results of HPLC analysis of finished compost showed significant accumulations of several TNT transformation products, TNB, 2ADNT, 4ADNT, and 4,4'AZOXY (Table 5).

Preparation of [¹⁵N]TNT-labeled compost. Recoveries of radioactivity in the four radiolabeled treatments averaged 101 ± 8.8 percent. The material from these reactors were frozen pending a need to fractionate them in support of the NMR analyses. Results of HPLC analyses revealed the formation of the following TNT transformation products by Day 20: TNB, 4ADNT, 2ADNT, 2,6DANT, 2,4DANT, and 4,4'AZOXY (Table 6). Lack of some of these products in the [¹⁵N]TNT treatments is unexplained.

Table 5 Results of HPLC Analysis of T₂₀ Compost (µg/g)					
	TNT	TNB	2ADNT	4ADNT	4,4'AZOXY
Treatments ¹	8.845 ± 1,100	31.4 ± 5.20	504 ± 282	1,258 ± 242	244 ± 125
Controls ²	0.70 ± 0.90	0.26 ± 0.02	0.32 ± 0.32	1.78 ± 0.81	<1.0
Detection Limits	0.10	0.10	0.10	0.10	1.00
¹ Values represent the mean of six replicates ± standard deviation.					
² Values represent the mean of two replicates ± standard deviation.					

Table 6 Concentrations of TNT and Its Transformation Products in Compost Prepared for ¹⁵N Analyses (µg per gram compost)¹								
Treatment	T ₀ TNT ²	T ₂₀ TNT	T ₂₀ TNB	T ₂₀ 4ADNT	T ₂₀ 2ADNT	T ₂₀ 2,6DANT	T ₂₀ 2,4DANT	T ₂₀ 4,4'AZOXY
¹⁵ N	11,800	883	<0.10	32.9	9.56	<0.50	<0.10	<0.10
¹⁵ N	15,600	1,050	<0.10	153	33.1	<0.50	<0.10	<0.10
Cold + ¹⁴ C	21,300	985	4.69	488	113	10.2	317	118
Cold + ¹⁴ C	32,900	3,080	7.30	1,340	249	26.0	630	125
Cold + ¹⁴ C	34,900	2,470	6.76	1,440	390	26.2	537	128
Cold + ¹⁴ C	33,300	1,840	8.85	1,680	868	15.8	126	141
Cold	32,000	5,020	15.9	1,420	740	10.5	216	129
Control	<1.00	<1.00	<0.10	<0.10	<0.10	<0.50	<0.10	<0.10
¹ Target concentration of TNT in compost at T ₀ was 20,000 µg per gram.								
² Only TNT was detected in T ₀ samples.								

Part II: NMR analyses

Solid-state CP/MAS ¹⁵N NMR spectra of pure [¹⁵N]TNT, the composted [¹⁵N]TNT-spiked soil sample before and after solvent extraction with acetonitrile, and the compost blank sample are shown in Figures 1-3. The peak corresponding to the nitro groups of pure [¹⁵N]TNT occurs at 366 ppm (Figure 1). The naturally abundant ¹⁵N nuclei in the control compost are observed in the peak at approximately 118 ppm (Figure 2). This peak corresponds primarily to the secondary amide nitrogens of amino acids in peptide structures. Although the naturally abundant nitrogens overlap with one of the major peaks of interest in the [¹⁵N]TNT compost (118 ppm) because the concentration of ¹⁵N label originating from the [¹⁵N]TNT is so much greater than the naturally abundant ¹⁵N nuclei in the compost, it is unlikely that the naturally abundant ¹⁵N nuclei contribute to the intensity of the peak at 118 ppm in the [¹⁵N]TNT compost spectra. The signal-to-noise ratio of the naturally abundant amide nitrogen peak in the blank compost developed at a

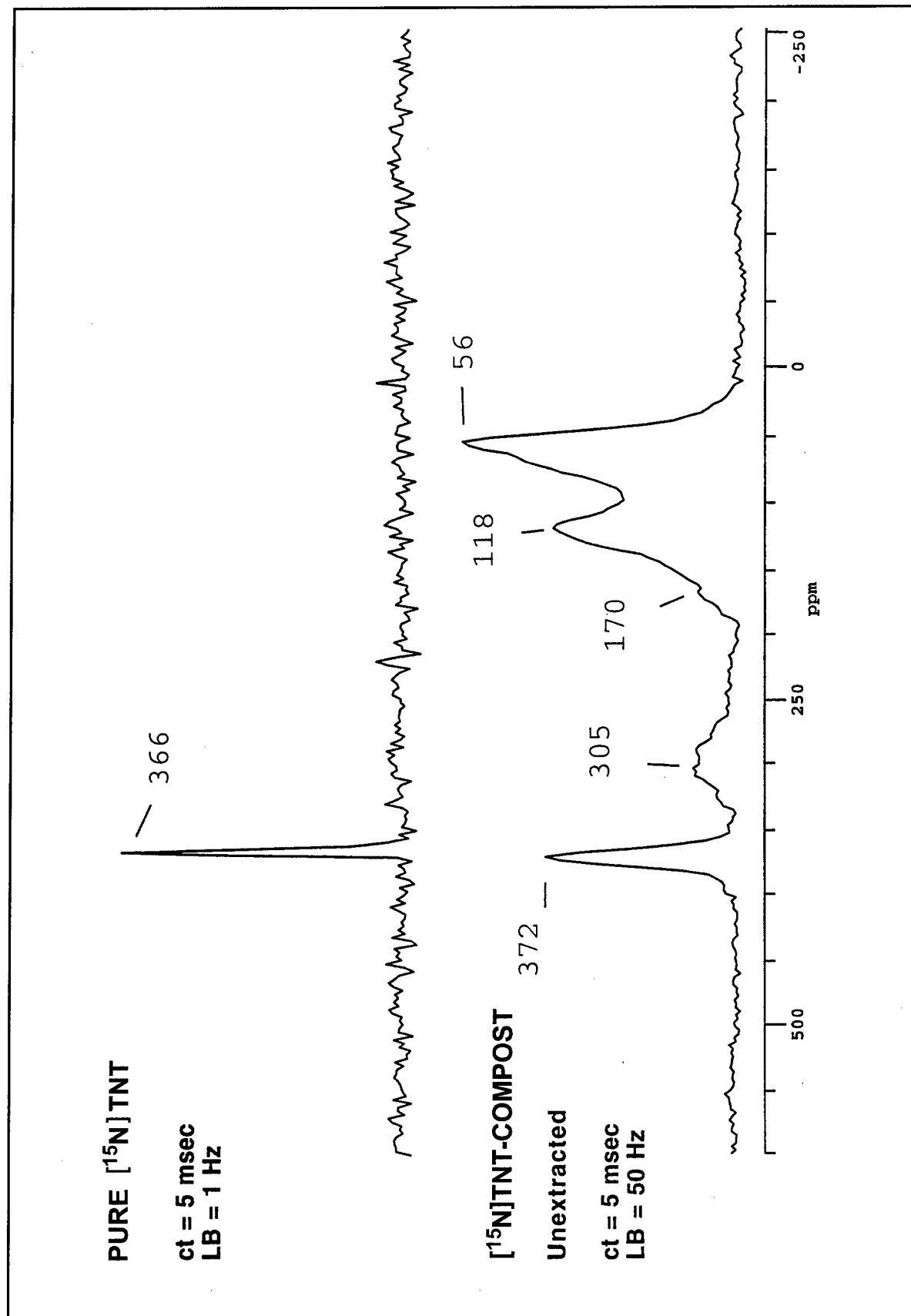


Figure 1. Solid-state CP/MAS ^{15}N NMR spectra

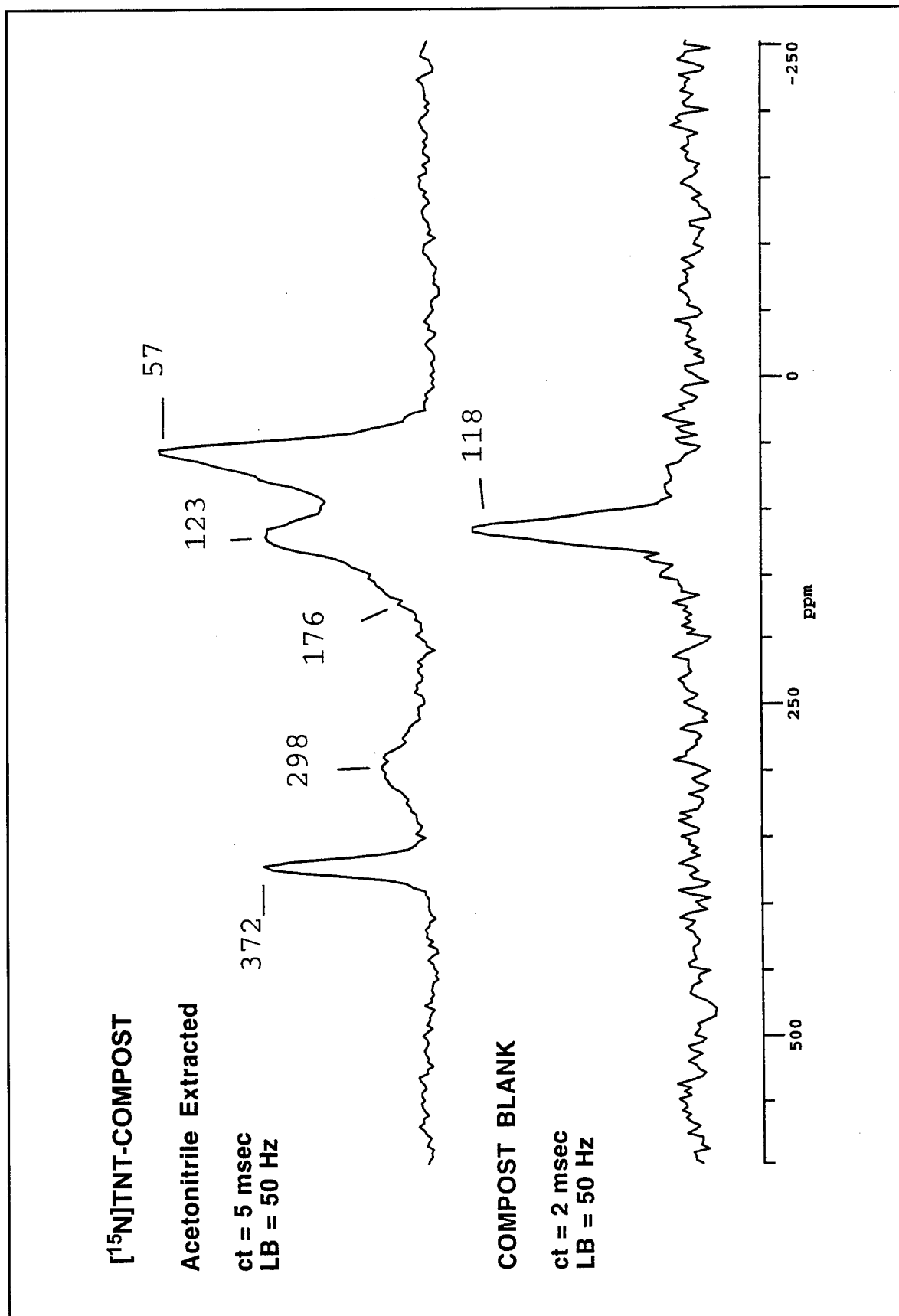


Figure 2. Solid-state CP/MAS ^{15}N NMR spectra

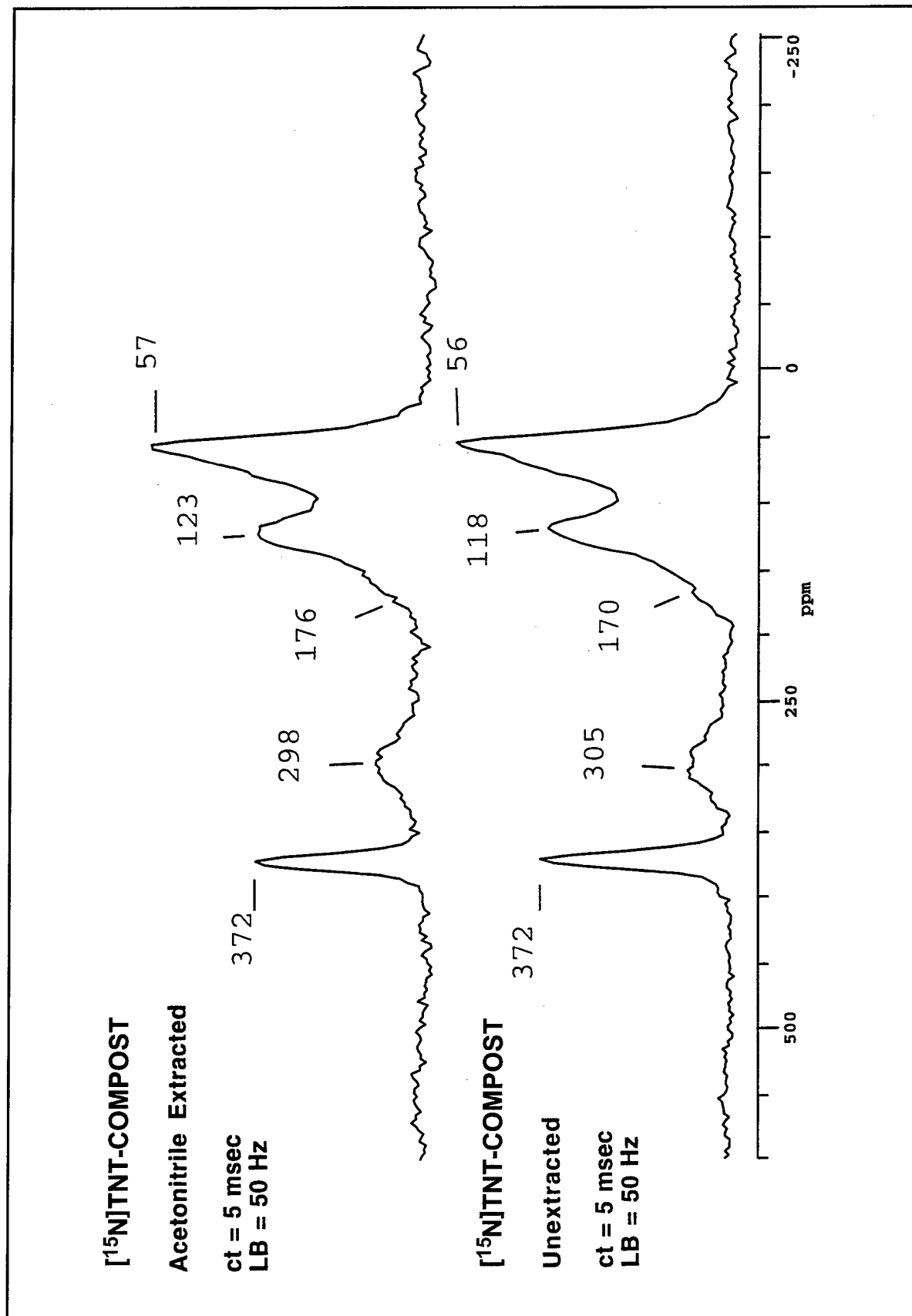


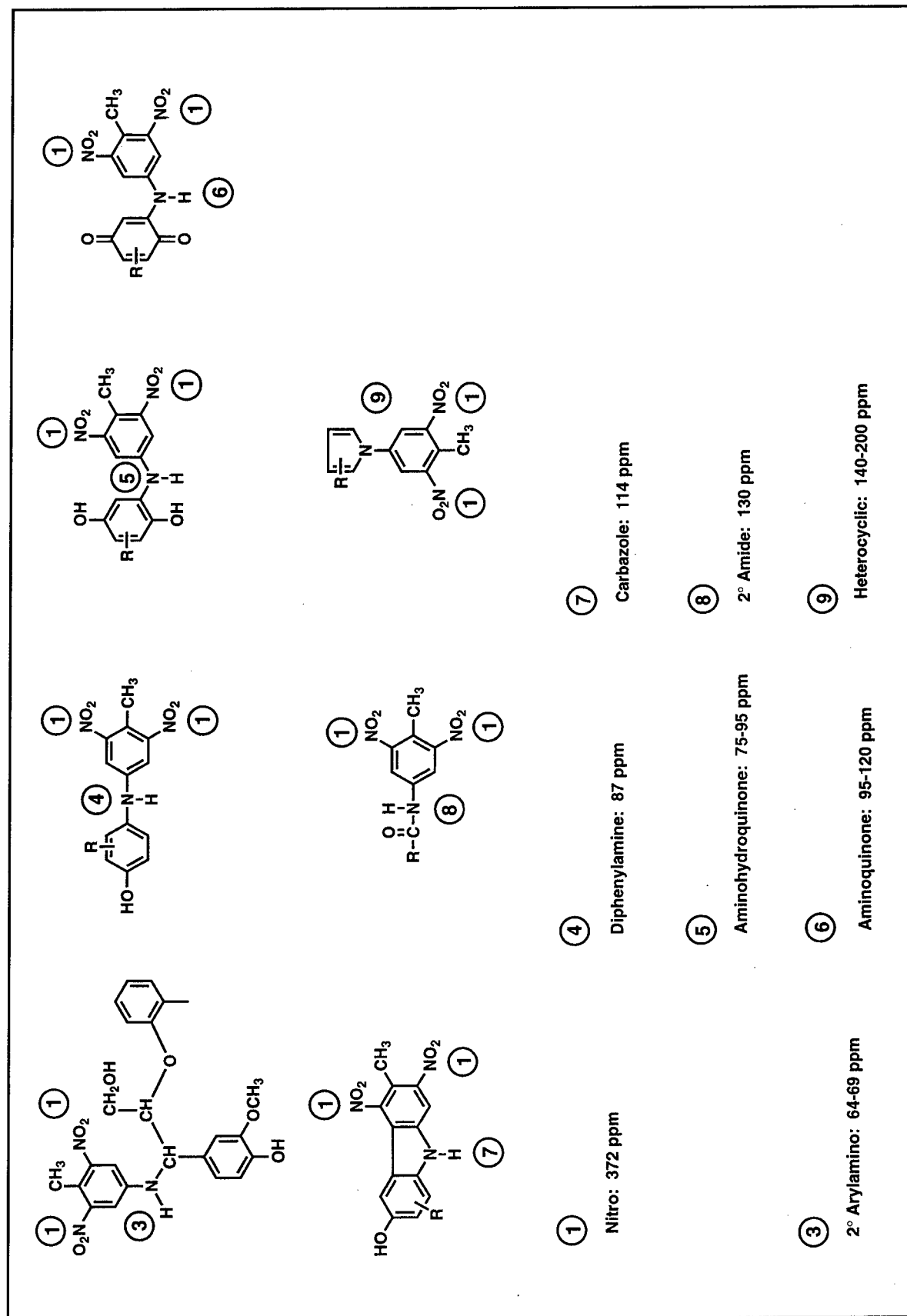
Figure 3. Solid-state CP/MAS ¹⁵N NMR spectra

significantly slower rate than the [^{15}N]TNT-derived peak at 118 ppm in the [^{15}N]TNT compost.

When comparing the spectra of the [^{15}N]TNT compost before and after solvent extraction, no discernible removal of labeled nitrogen with the acetonitrile can be observed (Figure 3). This is consistent with results listed in Table 6, where the amount of TNT extracted from the compost after 20 days incubation is less than the amount immobilized, by an order of magnitude. It can be assumed therefore that the peaks in the spectra of the [^{15}N]TNT compost represent transformation products covalently bound to organic matter. The spectra of the [^{15}N]TNT compost bear a resemblance to the spectra of the individual monoamines and diamines reacted with soil humic acid and peat, reported previously (Thorn 1998).

Once bonded to organic matter, each molecule of the monoamines, 2ADNT or 4ADNT, derived from the reduction of labeled [^{15}N]TNT would give rise to two separate peaks. One peak represents the bonded amine N and the other peak the residual nitro groups. The approximate chemical shift positions for the possible types of covalent bonds formed between the monoamines and organic matter, using 4ADNT as the example, are illustrated in Figure 4. Each molecule of 2,4DANT or 2,6DANT derived from reduction of [^{15}N]TNT potentially gives rise to three separate peaks. The three peaks would correspond to the reduced nitro group, the free amino group, and the covalently bonded amino group. These possible configurations are illustrated in Figure 5, using 2,4DANT as the example. Each molecule of TAT derived from [^{15}N]TNT would give rise to two peaks, corresponding to the two free amino groups and the covalently bonded amino group (Figure 6). Strongly reducing conditions ($E_h < -200$ mV) are required for complete reduction of TNT to TAT. Formation of TAT was not measured in the composting experiments, and so it is not known if production of TAT was significant enough for binding of TAT to occur.

Four major peaks of broad intensity occur from approximately 40 to 100 ppm, 100 to 200 ppm, 250 to 350 ppm, and 355 to 390 ppm in the spectra of the [^{15}N]TNT compost. The peak at 57 ppm corresponds to the free amine nitrogens of either the diamines or TAT bonded to organic matter. Downfield from this peak and extending to about 100 ppm are the secondary amine nitrogens of monoamines, diamines, or TAT bonded to organic matter through aminohydroquinone, enamine, hydrazine, and diphenylamine linkages. The free primary amine nitrogens are not completely resolved from the secondary amine nitrogens that are involved in covalent linkages to organic matter. The broad peak from 100 to 200 ppm can be approximately subdivided into singly protonated nitrogens (100-140 ppm) and nonprotonated nitrogens (140 to 200 ppm). Nitrogens of the monoamines, diamines, or TAT bonded to organic matter through aminoquinone, carbazole, or ester linkages occur from 100 to 140 ppm. Nitrogens of the monoamines, diamines, or TAT bonded to organic matter through heterocyclic linkages occur from approximately 140 to 200 ppm. The broad peak with maximum at 298 to 305 ppm corresponds to nitrogens of the diamines or TAT bonded to organic matter through imine linkages. Azoxy nitrogens also occur in this region. The peak at 372 ppm represents the nitro groups of the monoamines or diamines

Figure 4. ^{15}N NMR chemical shift ranges for adducts of monoaminos

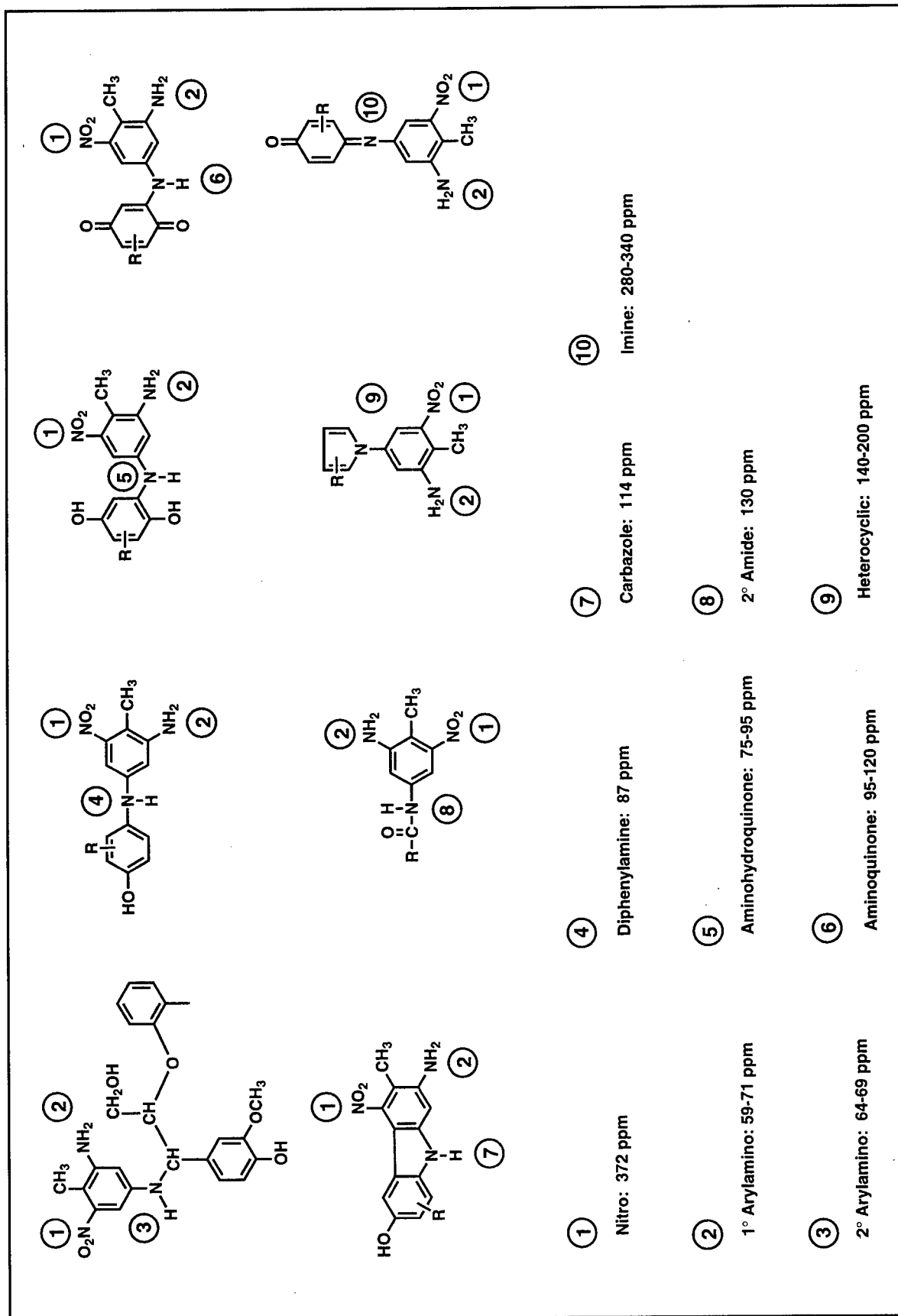
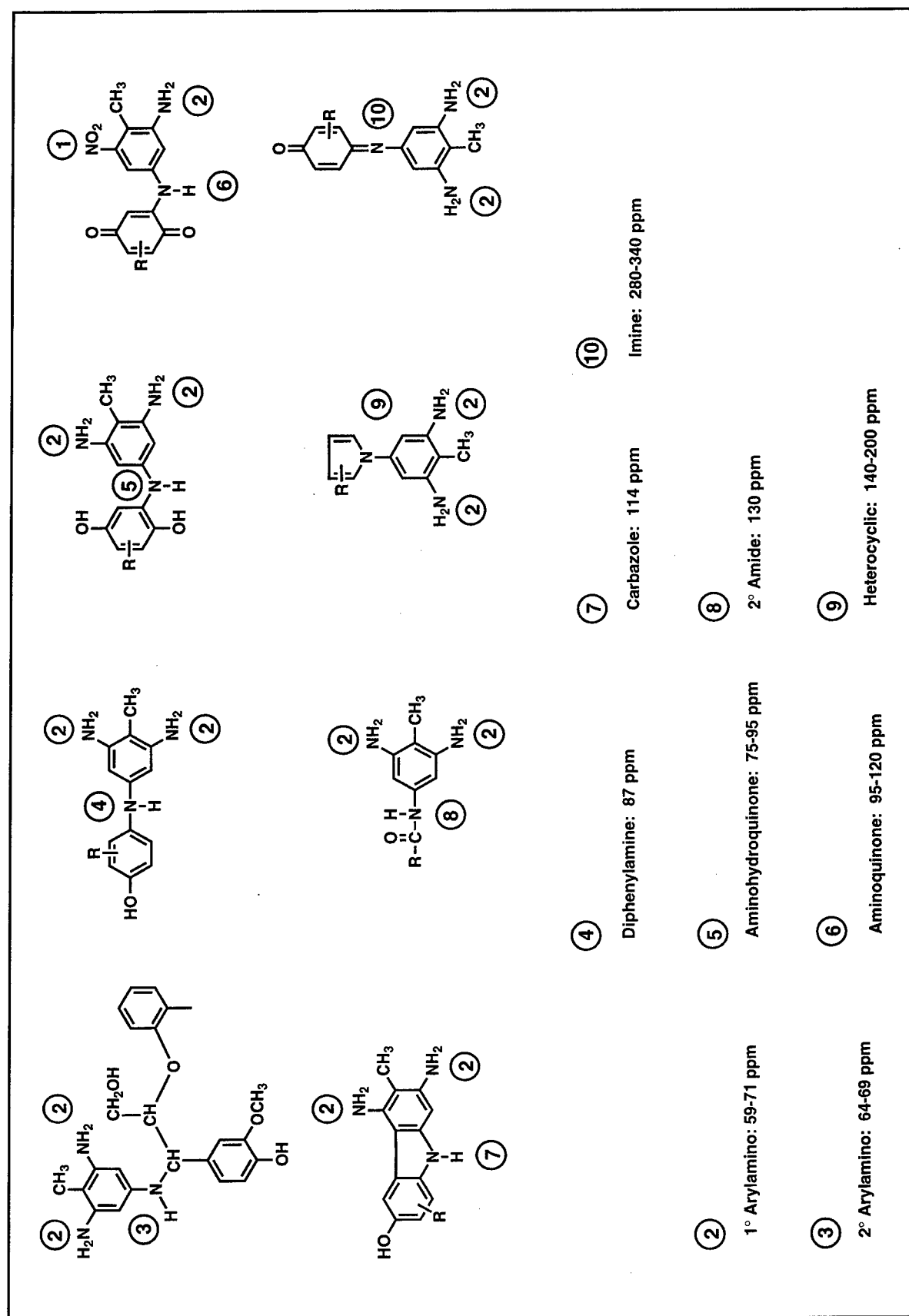


Figure 5. ^{15}N NMR chemical shift ranges for adducts of diaminos

Figure 6. ^{15}N NMR chemical ranges for adducts of TAT

covalently bonded to organic matter. No signals are present downfield from the nitro groups at 372 ppm through 1,000 ppm. Thus, with the signal-to-noise ratio attained, nitroso nitrogens (chemical shift range 410 to 920 ppm) and azobenzene nitrogens (chemical shift range 470 to 530 ppm) cannot be observed. A summary of ^{15}N NMR chemical shifts representing condensation products of aromatic amines with organic functional groups is reproduced in Figure 7 (Thorn et al. 1996a,b).

The relative amounts of monoamines versus diamines versus TAT that undergo covalent binding to organic matter cannot be directly determined from the ^{15}N NMR spectra. The possibility that the residual nitro groups of the bound monoamines or diamines undergo subsequent reduction cannot be discerned either. Several features of the spectra of the TNT compost do provide evidence for binding by the individual metabolites, based upon results from the binding studies of the individual amines with model compounds and humic acid. The presence of the nitro groups at 372 ppm constitutes evidence for binding by the monoamines or diamines, as bound TAT would not contribute to this signal. The presence of the free amino nitrogens at ~ 58 ppm constitutes evidence for binding of the diamines or TAT. The bound monoamines would not give rise to this signal. This latter point is illustrated in the spectra of the IHSS soil humic acid reacted with the monoamines and diamines (Figures 8 and 9; reproduced from Thorn 1998). The spectra from reactions of humic acid with the diamines exhibit upfield peak maxima at 59 ppm. In contrast, the upfield peak maxima from reactions of the monoamines occur at 68 to 79 ppm.

The diamines, 2,4DANT and 2,6DANT, were observed to undergo 1,2-addition to quinone groups in soil humic acid resulting in the formation of significant amounts of imine bonds. Birnessite and horseradish peroxidase (HRP) catalyzed an increase in the formation of imine bonds at the expense of heterocyclic condensation products. The monoamines, 4ADNT and 2ADNT, did not form imine bonds with soil humic acid in the absence of catalysts and only trace amounts in the presence of HRP. The significant concentration of imine nitrogens in the compost, therefore, constitutes evidence for covalent binding of the diamines and possibly for the involvement of phenol oxidase or metal catalysts in the binding of the diamines during composting.

Summary and Conclusions

The solid-state ^{15}N NMR spectra provide direct confirmation for the reductive degradation of TNT followed by covalent binding to organic matter during composting of the spiked soil. In general, the spectra are consistent with results from studies on the covalent binding of the individual monoamines and diamines with soil humic acid and peat. Several features of the spectra indicate covalent binding by the diamines as a significant process in the transformation of TNT into bound residues. The reduced TNT amines bind to organic matter through a broad variety of covalent bonds, including aminohydroquinone, aminoquinone, heterocyclic, and imine linkages.

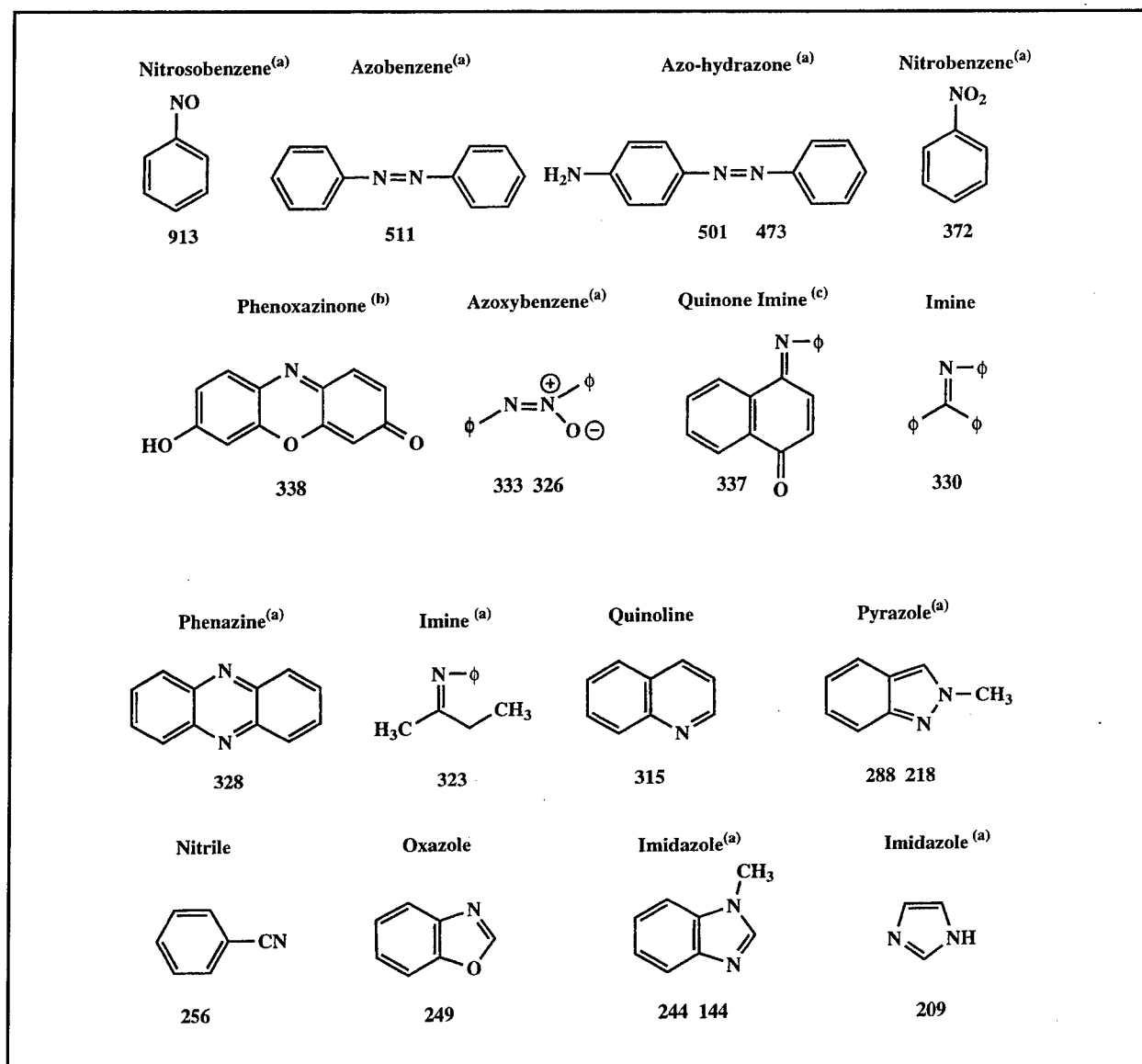


Figure 7. ¹⁵N NMR chemical shifts of nitrogen compounds representing condensation products of aniline with carbonyl and other functional groups (Chemical shifts determined in DMSO-d₆ in this laboratory unless otherwise noted. (a) References listed in Thorn et al. 1996a,b. (b) Determined in solid state. (c) Tentative assignment) (Continued)

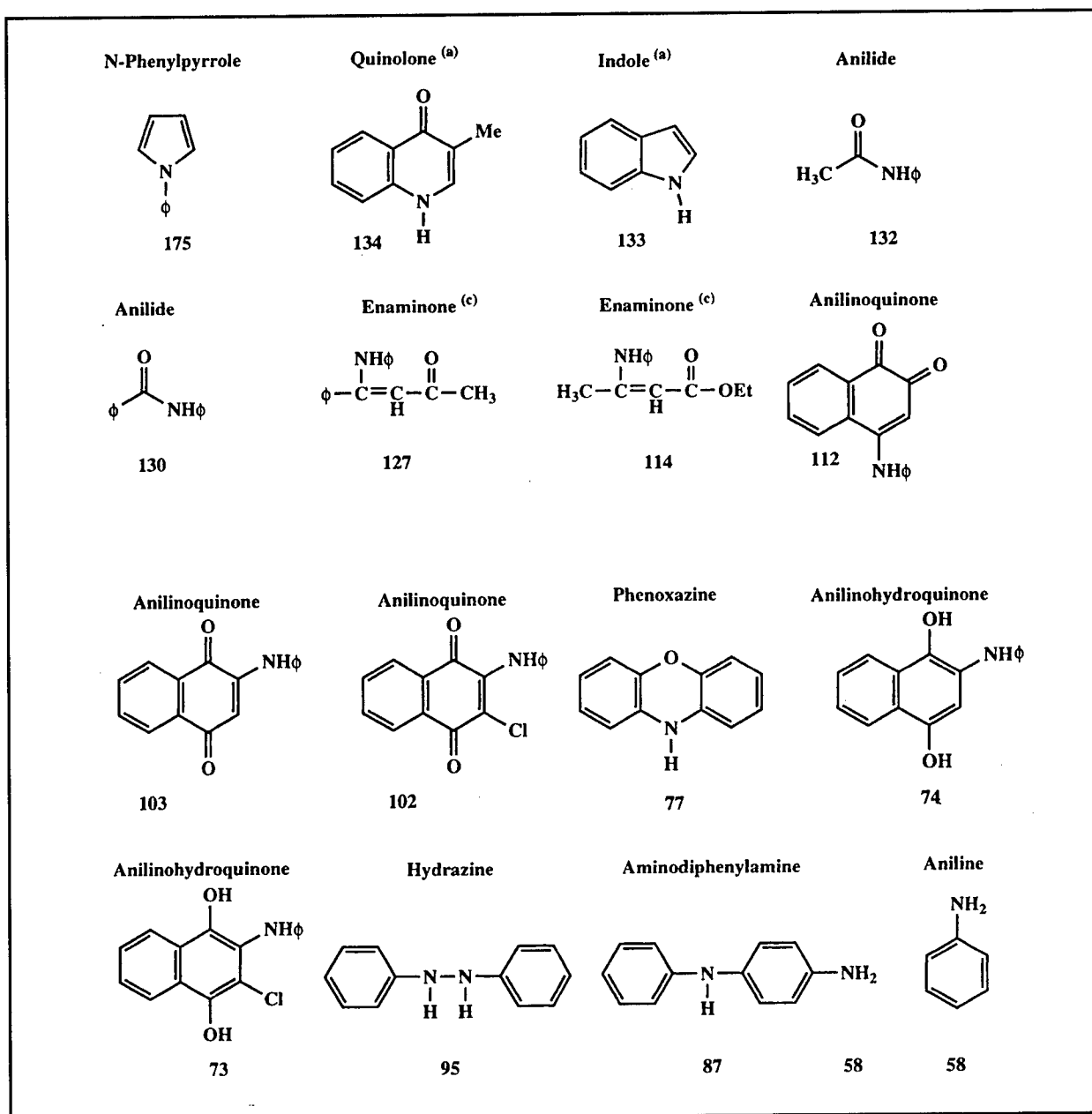


Figure 7. (Concluded)

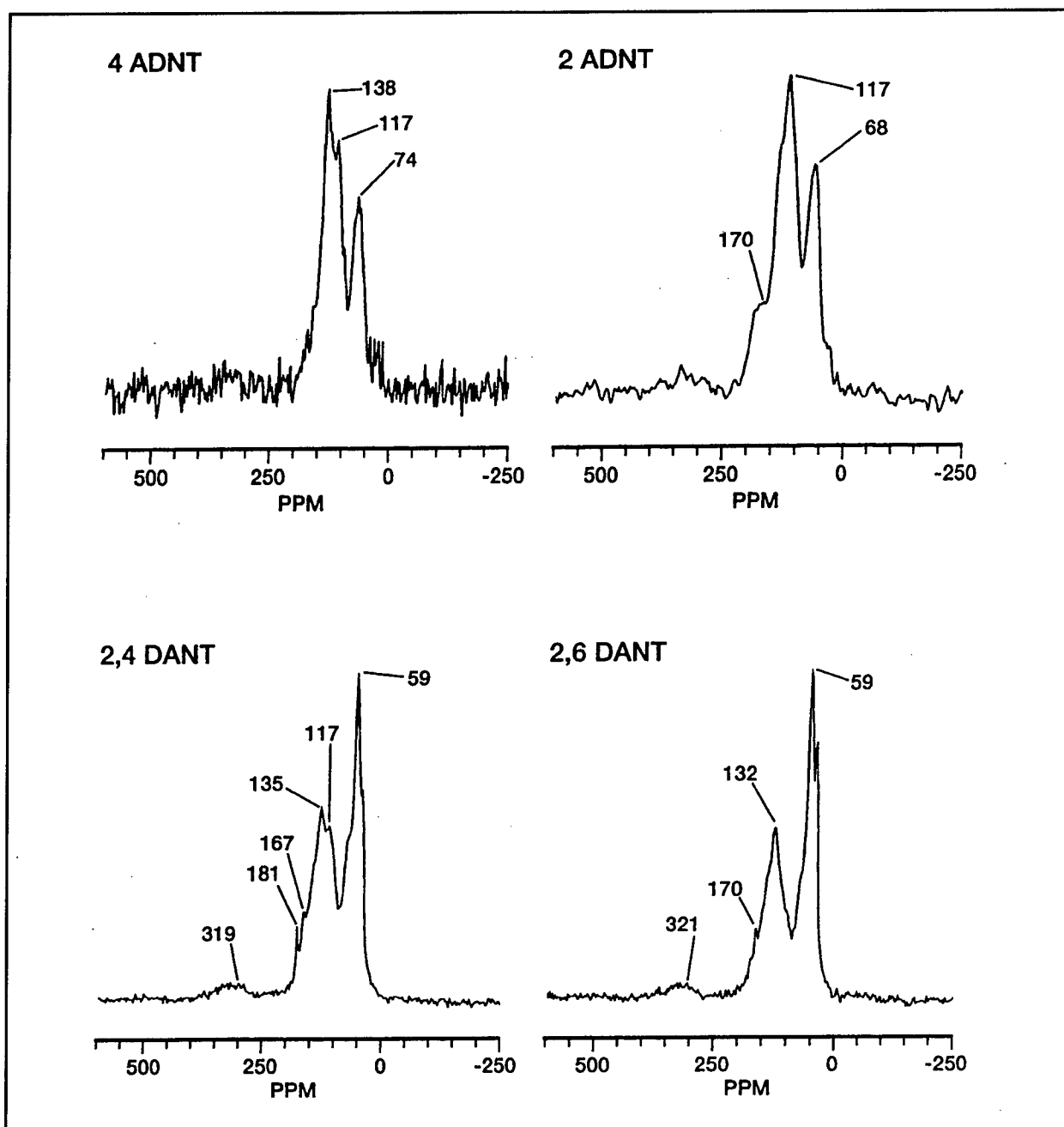


Figure 8. Solid-state CP/MAS ^{15}N NMR spectra of IHSS soil humic acid reacted with monoamines and diamines (No catalyst)

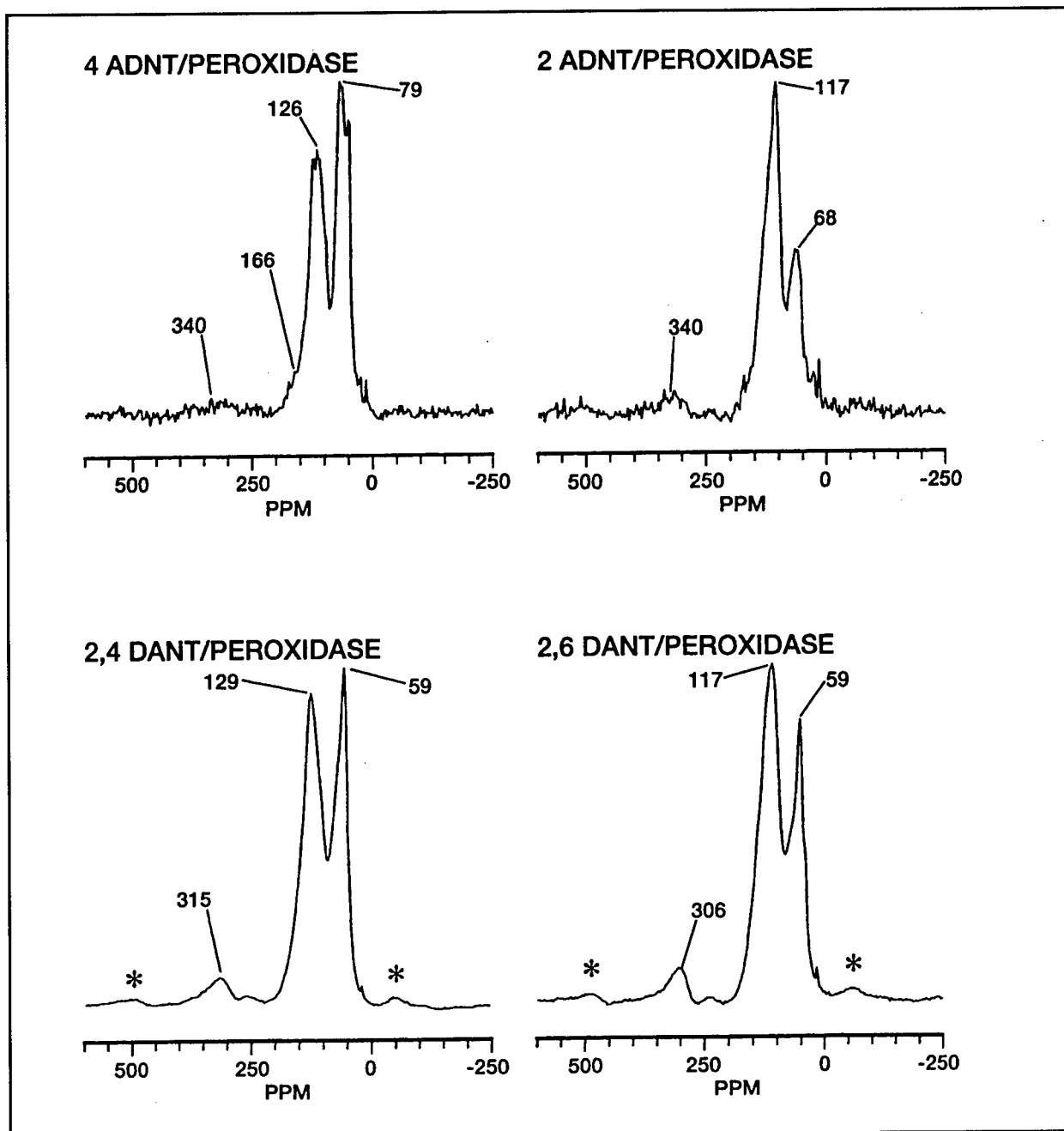


Figure 9. Solid-state CP/MAS ^{15}N NMR spectra of IHSS soil humic acid reacted with monoamines and diamines with horseradish peroxidase as catalyst

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3 Toxicity of Uncomposted and Composted Explosives-Contaminated Soils to Earthworms

Introduction

In previous investigations, survival, growth, reproduction, avoidance, and a battery of biochemical markers of exposure were monitored in earthworms, *Eisenia foetida*, exposed to soil spiked with TNT, RDX, or HMX and to the TNT-spiked soil after it had been composted (McFarland et al. 1998). Acute toxicity was tested with serial dilutions of the explosives in artificial soil. A 14-day LC_{50} of approximately 350 ppm was found for TNT in artificial soil, while no lethality occurred in either RDX- or HMX-spiked artificial soil at concentrations as high as 4,000 ppm. Exposure concentrations of 200 ppm for TNT and 400 ppm for RDX and HMX were selected for the 21-day chronic and sublethal effects tests: survival for all treatments was 100 percent. Although TNT-, HMX-, and RDX-spiked soils caused no mortality and were not genotoxic, the explosives caused a complete cessation of reproduction and failure to grow, altered the levels of some enzymes and substrates involved in protection against oxidative stress, and impaired immunocompetence. However, RDX and HMX did not invoke an avoidance response even at the highest concentration tested (4,000 ppm), while TNT invoked avoidance behavior even at levels as low as 25 ppm. Thus, worms may avoid areas contaminated with TNT and therefore avoid toxicity, while areas contaminated with RDX or HMX would not be avoided, and exposure would occur.

Composting of TNT increased toxicity, with only 35 percent of the worms surviving to Day 21 in the TNT finished compost as compared with 96 percent in the control compost. Survivors were insufficient in numbers in the TNT finished compost to statistically compare growth with that in the control compost, and reproduction was zero in both composted treatments: i.e., no cocoons or juvenile worms were found. The toxicity of the composted mixture could not be attributed to TNT, as less than the detection limit of 0.1 ppm remained. However, chemical

analysis of the composted mixture revealed the presence of 4.55 ppm of 4ADNT. Therefore, the residual toxicity of the compost was suspected to be attributable to metabolites of TNT.

Because of these effects, questions remained about the toxicity of explosives and the breakdown products of TNT in soils and composts. Therefore, further investigations were undertaken (a) to determine whether metabolites of TNT produced during composting could cause reduced survival of earthworms and (b) to find the lower limits for toxic effects of individual explosives concentrations in soils.

Materials and Methods

Experimental chemicals

Six metabolic degradation products of TNT (4,4',6,6'-tetranitro-2,2'-azoxytoluene (2,2'AZOXY); 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'AZOXY); 2-amino-4,6-dinitrotoluene (2ADNT); 4-amino-2,6-dinitrotoluene (4ADNT); 2,4-diamino-6-nitrotoluene (2,4DANT); and 4-hydroxylamino-2,6-dinitrotoluene (4HADNT)) were custom synthesized by Dr. Ron Spanggord of SRI International (Menlo Park, CA). Purified RDX was supplied by Dr. Stan Caulder of the Naval Surface Warfare Center, Indian Head Division, Indian Head, MD. TNT, HMX, and RDX were obtained from weapons-grade stocks in storage at the ERDC.

Experimental organisms

Earthworms, *Eisenia foetida*, were maintained in continuous culture from stocks obtained from Carolina Biological Supply Company (Burlington, NC). Worms were kept in moistened sphagnum peat and fed *ad lib* on a diet of Magic Worm Food (Carolina Biological Supply Co.) Young adults weighing 0.25-0.40 g live weight were selected for all experiments.

Contact toxicity

The LD₅₀s of the TNT metabolites were determined using a filter paper contact toxicity method adapted from Roberts and Dorrough (1984). Briefly, glass scintillation vials (25 by 45 mm) were lined with Whatman No. 1 filter paper (9-cm circular filter paper or 63.6-cm², folded and fitted into the vial). Chemicals were dissolved in acetone, and 1 ml of the solutions was pipetted onto the filter paper in the vial. The acetone was driven out using a stream of nitrogen applied for 3 min and vials were allowed to air-dry overnight. Prior to adding one worm per vial, 1 ml of water was added to moisten the paper. Controls received 1 ml of acetone, and chemical concentrations were expressed as milligrams per square centimeter, ranging from 0.0001 to 0.1 mg/cm², or

actual acetone solution concentrations from 6.36 mg/ml to 0.00636 mg/ml. Containers were then covered with plastic wrap with several pinholes for aeration, placed in a cardboard box, and kept on their sides in the dark for 48 hr. Worms were considered dead only if they were unable to respond upon gentle prodding of their anterior end.

Soils

Soils used, soil spiking, and the addition of composting amendments were as described in Pennington et al. (1998) with the exception that hay was substituted for alfalfa in the composting amendments. Initial exposures were conducted at a nominal soil concentration of 250 ppm TNT and 500 ppm RDX or HMX. For end points showing an effect, exposure concentrations were halved and continued in serial dilutions until no effect was observed. Duration of the exposures was 21 days.

Survival, growth, and reproduction

Exposures of earthworms for survival, growth, and reproduction assessments were conducted using modifications of the methods of Gibbs, Wicker and Stewart (1996) as previously described (McFarland et al. 1998). Briefly, earthworms were exposed in ziplock bags containing 100 g of soil or compost. Twenty bags were prepared for each treatment. Worm pairs were preweighed before being placed into the bags, and worm pair weights were again taken after 21 days for the surviving worm pairs to measure growth. Bags in which both worms survived were resealed and left for another 35 days, after which they were examined for the presence of cocoons (eggs) and juvenile worms to assess reproductive success. A second group of 10 bags were prepared at each treatment level and worm pairs added for the biomarker assays. After 21 days, the worms were placed in cryovials, frozen in liquid nitrogen, and stored in a -80 °C freezer until analysis.

Sublethal effects

Avoidance of contact, glutathione content (total, oxidized, and percent oxidized), enzymatic biomarkers of oxidative stress (protein content and enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione-S-transferase (GST), and glutathione peroxidase (GPX)), immunotoxicity (extruded cell count, nitroblue tetrazolium dye (NBT) reduction assay), and DNA single-strand breakage were all tested using methods previously detailed in McFarland et al. (1998). Exposures were conducted for 5 days and for 21 days for glutathione content and enzymatic biomarkers of oxidative stress, 21 days for immunotoxicity and DNA single-strand breakage, and 4 days for avoidance behavior.

Data analysis

Normality was tested using Shapiro-Wilk's test and equality of variances using Levene's test, and in all cases data were transformed by conversion to rankits (normalized ranks) (SAS Institute, Inc. 1988). One-way analysis of variance was used, and means were compared by Dunnett's test. Student's t-test was used to compare means of the immunotoxicity tests and in the avoidance bioassay.

Results and Discussion

TNT metabolites and toxicity of composted TNT

Contact toxicity of TNT metabolites. The azoxy compounds, 2,2'AZOXY and 4,4'AZOXY, were relatively nontoxic, with LD_{50} s greater than 0.1 mg/cm^2 . Two metabolites, 2ADNT and 2,4DANT, had LD_{50} s between 0.1 and 0.01 mg/cm^2 . Both 4HADNT and 4ADNT had LD_{50} s between 0.01 and 0.001 mg/cm^2 (Table 1), with 4ADNT being the more toxic.

Table 1				
Percent Survival of Earthworms in Contact Toxicity Test				
Compound	Concentration, mg/cm^2			
	0.1	0.01	0.001	0.0001
2,2'AZOXY	80	100	100	100
4,4'AZOXY	100	100	100	100
2ADNT	40	89	100	100
4ADNT	(not tested)	0	100	100
2,4DANT	0	78	100	100
4HADNT	0	22	100	100

Note:
2,2'AZOXY is 4,4',6,6'-tetranitro-2,2'-azoxytoluene.
4,4'AZOXY is 2,2',6,6'-tetranitro-4,4'-azoxytoluene.
2ADNT is 2-amino-4,6-dinitrotoluene.
4ADNT is 4-amino-2,6-dinitrotoluene.
2,4DANT is 2,4-diamino-6-nitrotoluene.
4HADNT is 4-hydroxylamino-2,6-dinitrotoluene.

Toxicity of 4ADNT in soil. In a previously reported exposure of worms to TNT compost (McFarland et al. 1998), 50 percent of the worms died in 8 days. The composted TNT was shown to contain primarily 4ADNT (4-5 ppm), and this was also the most toxic of the breakdown products in the contact toxicity testing. According to Reinecke (1992), a contact toxicity LD_{50} value between 0.01 and 0.001 mg/cm^2 would correspond roughly to 10 ppm in soil. Artificial soil was spiked with 4ADNT at 300 ppm, and a serial dilution from 30 to 0.3 ppm was tested. Worms were exposed as described in the EPA earthworm acute toxicity method (Greene et al. 1989) for 14 days. No worms died during the exposure period, indicating that the level of 4ADNT (4-5 ppm) previously observed in composted soil was well below the LD_{50} . The conclusion drawn was

that no metabolite of TNT could have been responsible for the toxicity observed in the finished TNT compost. Because the amended TNT-spiked soil prior to composting was rapidly lethal to 100 percent of the earthworms, suspicion was now placed on the amendments as the source of toxicity. This was supported by experiments in which alfalfa was lethal to earthworms. Effects of 4ADNT on growth reproduction and ssDNA strand breaks were used to evaluate potential chronic and sublethal toxicities. No significant differences were observed between controls and 30 ppm of 4ADNT in artificial soil for any of the assays.

Toxicity of TNT compost. Artificial soil was spiked with TNT and composted as previously reported (Pennington et al. 1998), but using hay as an amendment instead of alfalfa. Significant TNT breakdown occurred even prior to composting. The initial TNT and 4ADNT concentrations were 20-30 ppm, even though the nominal TNT spiking level was 200 ppm. Breakdown of TNT may have been due to the replacement of alfalfa with hay, which resulted in a large mushroom bloom not observed in earlier composting runs. Since many fungal species are capable of extensive biotransformation of xenobiotics, the hay-associated fungus may have been responsible for the rapid TNT breakdown. Although test conditions were different from those under which toxicity had been observed earlier, the products were similar in chemical constituents and in concentrations (~3 ppm of 4ADNT and ~0.5 ppm of 2ADNT). The lack of acute toxicity to the earthworm confirmed that the toxicity previously observed was due to the alfalfa rather than to the metabolites of TNT formed during the composting process.

LOAEL and NOAEL determinations for TNT, RDX, and HMX in soil

Growth and reproduction. In the first set of exposures, decreases in cocoon production were observed at 200 ppm TNT, 250 ppm RDX, and 250 ppm HMX. The only significant weight change was observed with 250 ppm HMX because of large variations in weight gains. In an attempt to decrease the variability, a second and third exposure set was run. The worms in these sets were chosen to have more uniform initial weights. For graphical comparison, weight gains of all sets were normalized to the control values: alterations in growth and reproduction appear to be highly dose related. From the combined data sets, lowest observed adverse effect levels (LOAELs) for growth were 100 ppm for TNT, 250 ppm for RDX, and 25 ppm for HMX. No observed adverse effect levels (NOAELs) for growth were 50 ppm for TNT, 100 ppm for RDX, and 10 ppm for HMX (Figure 10). Lower doses appeared to have a slightly positive effect on growth for all compounds and on growth for TNT and RDX. For reproduction, LOAELs were 200 ppm for TNT, 250 ppm for RDX, and 25 ppm for HMX, and NOAELs were 100 ppm for TNT, 125 ppm for RDX, and 10 ppm for HMX (Figure 11). Problems with this assay because of variability in weight change between runs were largely eliminated by selecting adult worms within a narrow initial weight range and by checking soil hydration levels carefully. LOAELs and NOAELs of the most sensitive assays are summarized in Tables 2 and 3.

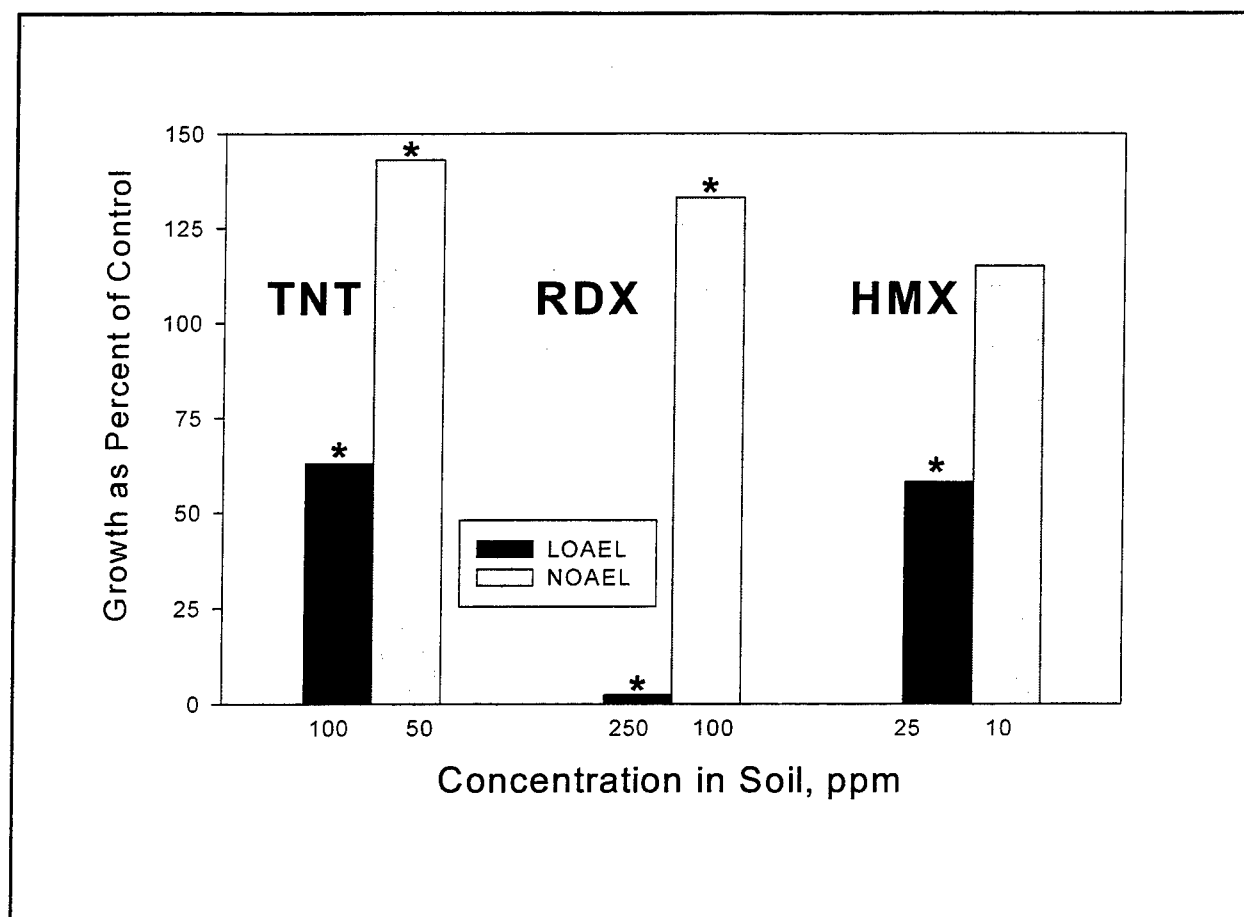


Figure 10. Lowest observed adverse effect levels (LOAELs) and no observed adverse effect levels (NOAELs) for effect on growth of earthworms as total dry weight change for individual explosives in artificial soil (Asterisk indicates significant difference from control, $P \leq 0.05$)

Avoidance assay. Initial avoidance tests indicated that of the three explosives tested (TNT, 200 ppm; RDX, 400 ppm; HMX, 400 ppm), the worms avoided only TNT. A TNT-dilution series was tested, and worms avoided the TNT-spiked soil down to 50 ppm TNT.

Immunotoxicity. All three compounds reduced the number of extruded cells, with a maximal reduction in extruded cell count of ~60 percent that of the control value. The NOAEL was 25 ppm for RDX, between 10 and 50 ppm for TNT, and 1 ppm for HMX (Figure 12). Clear LOAELs of 50 and 5 ppm were obtained with RDX and HMX, respectively, with all higher concentrations producing significantly reduced numbers of extruded cells relative to controls. In the case of TNT, a clear dose-related response was not observed in that immunotoxicity was significantly greater at 25 ppm than at 50 ppm, most likely because of the high variability in the 25-ppm exposures. Although all three of the explosives adversely affected the immune system of the earthworm by decreasing the number of extruded coelomocytes (white blood cell equivalents), no trend was observed in

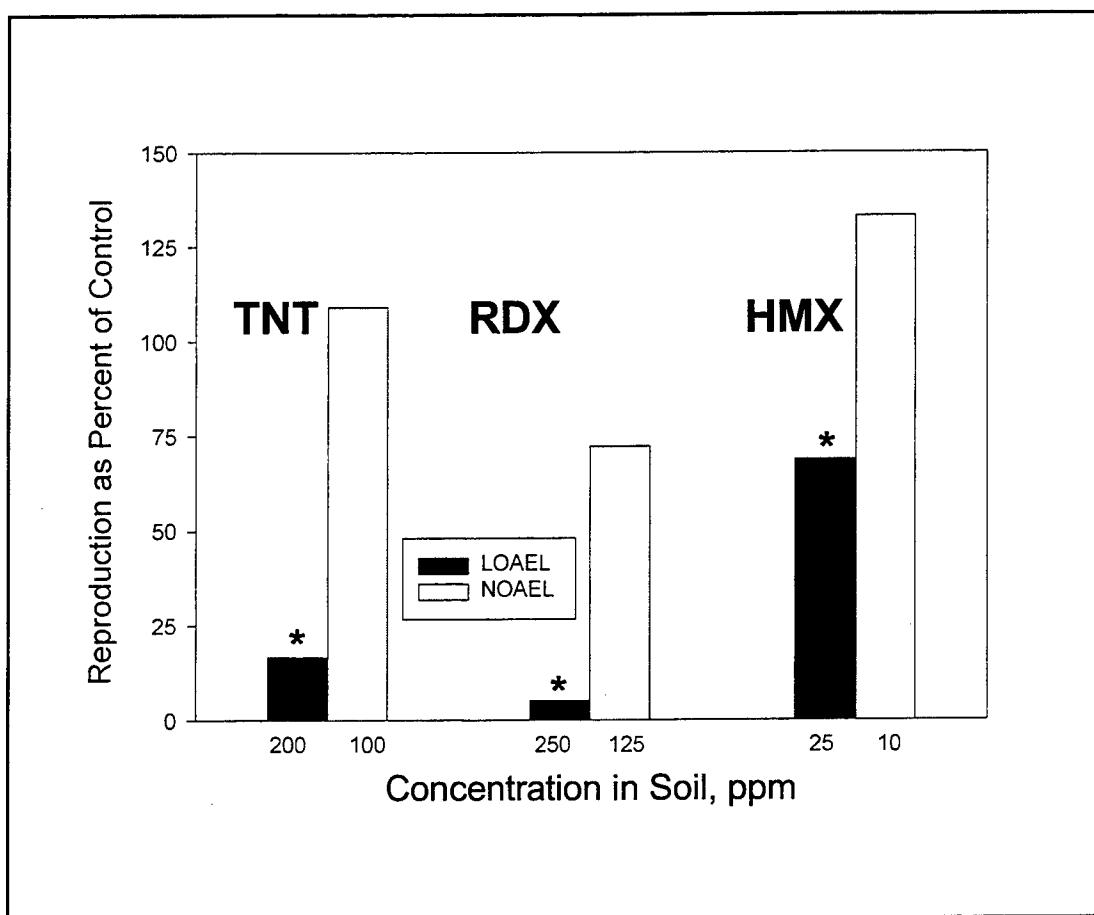


Figure 11. Lowest observed adverse effect levels (LOAELs) and no observed adverse effect levels (NOAELs) on reproduction of earthworms as a percentage of control cocoon production for individual explosives in artificial soil (Asterisk indicates significant difference from control, $P \leq 0.05$)

Table 2 Summary of LOAELs¹ for Most Sensitive End Points (Weapons-grade explosives in artificial soil; concentrations in ppm dry weight)			
End Point	TNT	RDX	HMX
Avoidance	50	>500	>500
Total glutathione (21-day)	200	250	100
Postmitochondrial protein (21-day)	10	50	10
Growth	100	250	25
Reproduction	200	500	25
Immunotoxicity	25	50	5

¹ LOAEL: Lowest observed adverse effect level.

Table 3
Summary of NOAELs¹ for Most Sensitive End Points (Weapons-grade explosives in artificial soil; concentrations in ppm dry weight)

End Point	TNT	RDX	HMX
Avoidance	25	≥ 500	≥ 500
Total glutathione (21-day)	100	100	50
Postmitochondrial protein (21-day)	5	25	5
Growth	50	100	10
Reproduction	100	250	10
Immunotoxicity	10	25	1

¹ NOAEL: No observed adverse effect level.

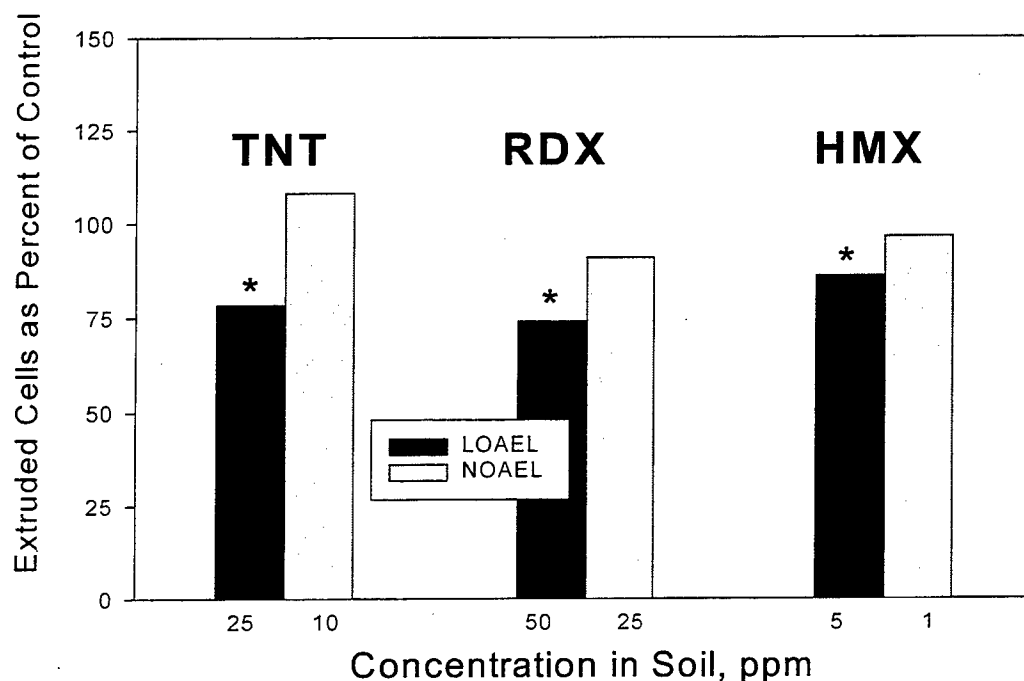


Figure 12. Lowest observed adverse effect levels (LOAELs) and no observed adverse effect levels (NOAELs) on immunocompetence of earthworms as a percentage of control extruded cells for individual explosives in artificial soil (Asterisk indicates significant difference from control, $P \leq 0.05$)

the NBT assays. Results were inconclusive with the A515 values occasionally higher (RDX 10 and 50 ppm, HMX 10 ppm) and occasionally lower (RDX 25 and 500 ppm, TNT 10 and 200 ppm) than that of their corresponding control values.

Antioxidant status: Enzymes and glutathione. Acute (5-day) exposure to TNT did not affect total glutathione or protein levels. The only significant effect observed for acute exposure to TNT was a decrease in glutathione peroxidase, which could be an indication of toxicity. Chronic (21-day) exposure to TNT resulted in significantly higher glutathione content in the 200-ppm TNT-exposed earthworms as compared with the control earthworms and significantly increased postmitochondrial protein levels (lowest observable effect level was 25 ppm, and the no observable effect level was 10 ppm).

The acute effects of HMX and RDX were similar. At high doses (500 ppm), antioxidant enzymes increased in the earthworm, either as a result of an overall, nonspecific increase in protein production or as an alteration in the specific activity of a particular enzyme (SOD). Total glutathione content was not affected at the 500-ppm level. However, at a slightly lower dose (250 ppm), while protein content and enzyme activities remained the same, total glutathione levels were significantly higher than those of controls. At much lower doses (10 ppm), total glutathione levels were actually lower than those of controls. Significant responses were observed at lower doses in the chronic tests than in acute tests. Protein content was elevated in worms exposed to midlevel doses of either RDX (50 and 100 ppm) or HMX (50 and 25 ppm) for 21 days; below these dose levels, no significant differences were observed for proteins or enzymes. Chronic exposure to RDX did not affect glutathione content at concentrations ranging from 50 to 500 ppm RDX. Mid to high levels of HMX (100 to 500 ppm) resulted in increased total glutathione, while lower levels (50 and 25 ppm) had no effect on glutathione.

The specific activity of protective enzymes can be selectively increased in response to a chemical insult without affecting total protein levels to a great extent. Alternatively, the protective response may be nonselective production of a wide array of proteins, which may include generalized stress response enzymes (heat-shock proteins), enzymes involved in biotransformation of xenobiotics (both Phase I and Phase II), or specialized enzymes such as the antioxidant enzymes (SOD, CAT, GPX) resulting in increased total protein levels. In both acute and chronic earthworm exposures, protein levels increased; selective induction of enzymes was only observed in acute exposures to midlevel doses of HMX and RDX. Earthworms seem to favor the nonselective increase in proteins in response to the explosives tested, especially under chronic exposure conditions. Increased postmitochondrial protein level after chronic explosives exposure was the most sensitive end point of the antioxidant enzyme assays.

Although the response of glutathione to chronic exposure to the explosives clearly indicated that HMX and TNT elevated total glutathione levels in treated compared with control animals, acute exposure to RDX and HMX resulted in

nonlinear responses; at high doses, total glutathione was elevated compared with controls; at middoses, glutathione was not significantly different from controls; while at low doses, glutathione was significantly lower in treated than in control animals. A possible explanation is that at the higher acute doses, oxidative stress is alleviated by the increased protection of SOD and/or the general increase in total proteins observed for both compounds at the higher doses (>250 ppm). At lower doses, increased glutathione synthesis plays a larger role, and glutathione content increases. At still lower doses, the synthesis of glutathione is not induced, and glutathione drops as it is consumed in antioxidant reactions.

The dissimilarities in responses to TNT exposure as compared with RDX and HMX indicate that the mechanisms of toxic action are different. This is supported by the fact that while TNT is acutely toxic ($LD_{50} \sim 350$ ppm), RDX and HMX are not acutely toxic at doses up to 5,000 ppm. TNT does not affect either glutathione or antioxidant enzyme status unless concentrations approach acutely toxic levels (200 ppm, which is approximately the LD_{10} dose). At this dose, a drop in GPX activity was observed after 5 days of exposure, possibly indicating toxicity (enzyme deactivation). The responses to acute exposure to HMX and RDX were similar to each other. However after long-term exposure to RDX, glutathione levels were not significantly different from controls, while long-term exposure to HMX resulted in increased glutathione content. These results suggest that while acute exposure to high levels of either RDX or HMX may generate oxidative stress, as indicated by the response of glutathione (GSH) and superoxide dismutase (SOD), only HMX elicits an antioxidant response in the earthworms upon chronic exposure.

While the information generated in these tests is valuable to an understanding of how explosives affect the biochemistry of the earthworm and help to elucidate possible mechanisms of action, these end points are not good choices as biomarkers of exposure to explosives. This is due to the complexity of the interactive relationships between glutathione and the antioxidant enzymes. Other, less specific end points such as effects on growth, reproduction, and the immune system provide more easily interpreted data with better application to risk assessment.

Toxicity of purified RDX. After the completion of the NOAEL exposure series for TNT, HMX, and RDX, the presence of approximately 10 percent HMX was discovered in the weapons-grade RDX stock (Pennington et al. 1998). For many of the biomarkers, the LOAEL for the weapons-grade RDX was approximately 10-fold higher than that of HMX. Therefore, the question arose regarding whether the toxicity observed may have been due to the HMX present in the impure weapons-grade RDX stock. In order to address this question, pure RDX was obtained, worms were exposed to 500 ppm in artificial soil for 21 days, and all previously described end points determined. No response was observed for enzymes or protein content (results identical to the contaminated RDX exposure), indicating that RDX does not affect the antioxidant enzyme system. Analysis of glutathione content indicated a significant reduction in oxidized glutathione (both in mmol/g wet weight and as a percent of the total glutathione content). This trend was also present in the 500-ppm weapons-grade RDX exposure, although the differences were not significant. Thus, RDX is capable of altering the glutathione

status of the organism. Growth and reproduction were about one-half that of controls in worms exposed to 500-ppm pure RDX. The effect on growth was markedly different in the pure RDX exposure from that in either the 50-ppm HMX or 500-ppm weapons grade RDX exposures (Figure 13). In the latter exposures, worms not only failed to grow but lost weight. Most of the effect originally observed with the weapons-grade RDX was due to the HMX contamination. No significant differences were observed for the immunoassay tests (extruded cell count) upon exposure to 500-ppm pure RDX, unlike the previous exposure to 500-ppm weapons-grade RDX in which a significant reduction in extruded cell count was detected. Interestingly, the 500-ppm weapons-grade RDX-exposed worms, which were exposed to 50-ppm HMX as an adulterant, had a similar response as the 50-ppm HMX-exposed worms. Furthermore, the LOAEL level for weapons-grade RDX (50 ppm, with 5-ppm HMX contamination) corresponded to the LOAEL level for HMX (5 ppm) (Figure 14). The toxicities observed for weapons-grade RDX in the earthworm toxicity tests was primarily due to the presence of HMX, not RDX, with the minor exception of a decrease in oxidized glutathione by RDX.

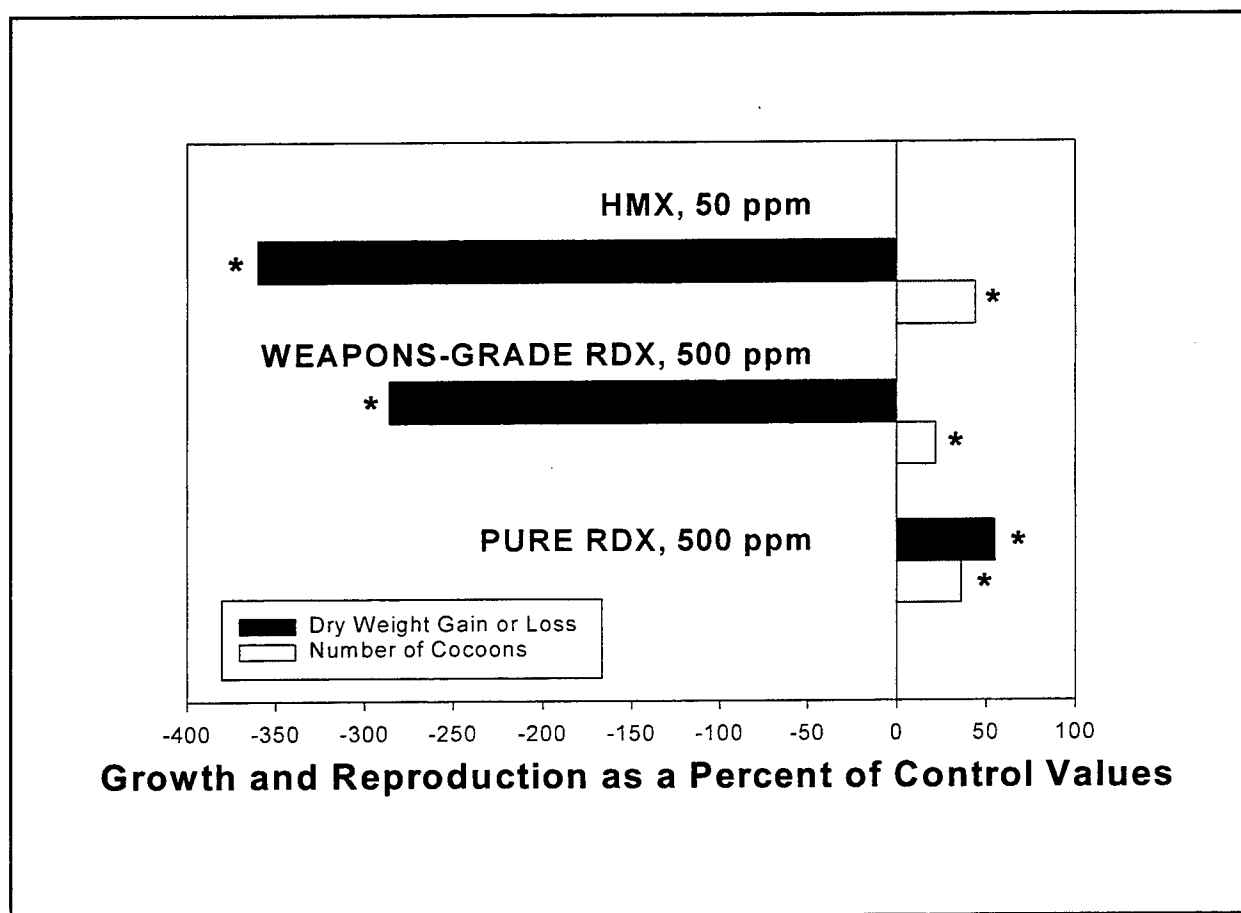


Figure 13. Effects of HMX, RDX, and purified RDX in artificial soil on earthworm growth and reproduction (Asterisk indicates significantly less than control, $P < 0.05$)

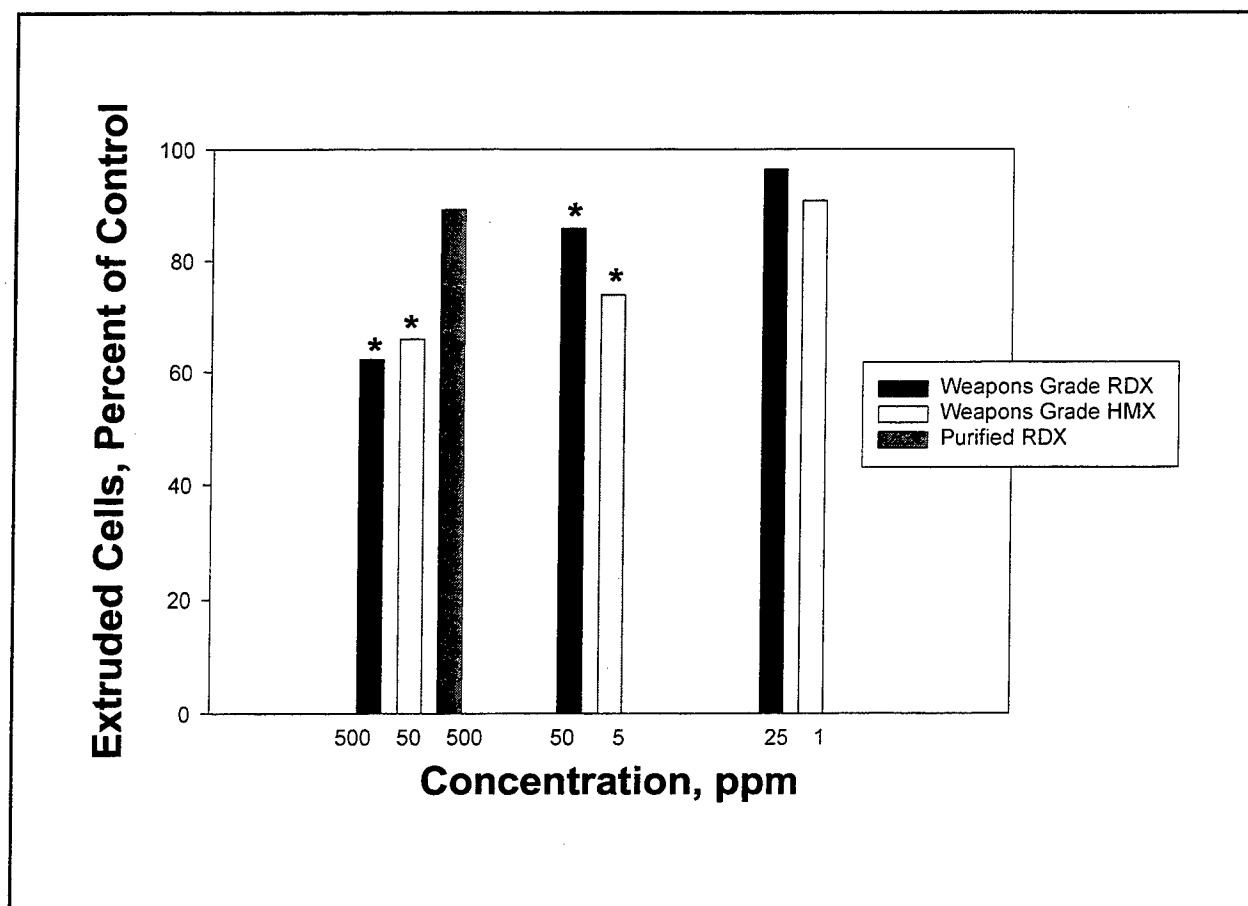


Figure 14. Effects of HMX, RDX, and purified RDX in artificial soil on earthworm immunocompetence (Extruded cells in exposed worms shown as a percentage of extruded cells in controls. Asterisk indicates significantly less than control, $P < 0.05$)

Conclusions

Residual toxicity to earthworms that remained in composted TNT-contaminated soil was found not to be due to metabolites of TNT formed during the composting process. Even the most toxic metabolite (4ADNT) determined in the earthworm contact toxicity test had no effect on survival, growth, reproduction, or any sublethal end point measured in the earthworms at concentrations in the range found in composted material. Alfalfa added as an amendment for composting was toxic to earthworms. When alfalfa was replaced with hay, the toxicity was eliminated.

RDX was essentially nontoxic. The toxicities previously observed for RDX (weapons-grade) were almost entirely the result of adulteration with HMX. This is an important point for bioremediation efforts; although more easily degraded, RDX is less toxic than HMX, which does not compost efficiently.

The six end points for which LOAELs and NOAELs (avoidance, total glutathione (21-day), postmitochondrial protein (21-day), growth, reproduction, and immunotoxicity) were determined are sensitive indicators of explosives contamination. These values can aid in the establishment of environmentally acceptable end points to be used in lieu of analytical chemistry data to establish cleanup and remediation levels of explosives in soils. Analytical chemistry is expensive and, in any case, not directly interpretable in terms of ecological effects. Conversely, biological end points such as growth, reproduction, and immunotoxicity are highly relevant and easily interpretable.

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4 Conclusions

Character of Immobilized Products

An understanding of the chemical interactions between TNT transformation products and functional groups on humic substances was achieved using liquid- and solid-state ^{15}N NMR analysis. Results indicated that the monoamino and diamino transformation products of TNT covalently bind to carbonyl compounds, humic substances, and whole peat in compost and soil. The number of potential products forming from the interaction of the diamino transformation products of TNT with humic functional groups is greater than the number from the monoamino transformation products. This is attributed to the higher pKa and greater nucleophilicity of the diamino products. Furthermore, the 4ADNT produces more products than the 2ADNT. This is due to steric hindrance effects in the 2ADNT.

The enzyme peroxidase failed to catalyze auto condensation of the monoamines. However, the enzyme catalyzed the formation of several products from 2,4DANT, some identified (e.g., azoxybenzenes, imines, hydrazines) and some uncertain. Fewer products were observed from 2,6DNT. Polymerization of coniferyl alcohol by peroxidase is a potential biosynthetic pathway for the formation of lignin in woody plants. Peroxidase catalyzed reactions of the monoamines and diamines with coniferyl alcohol resulting in complex products. These results demonstrate the possibility of such reactions in plants.

Reactions between the diamines and humic acid in the presence of the enzyme resulted in greater imine formation than heterocyclic nitrogen formation. Deamination with release of ammonia was also observed. In the absence of the enzyme, the relative proportion of imines and ammonia was less. Reactions between the monoamines and humic acid result in more heterocyclic products than imines. The enzyme also enhanced these reactions.

Spectral analyses before and after hydrolysis of TNT that had been reacted with peat indicated that some bonds were not hydrolyzable. Aminoquinone, amide, and imine linkages were hydrolyzable, while aminohydroquinone bonds persisted. Heterocyclic products were resistant to hydrolysis.

Analysis of whole compost by solid-state ^{15}N NMR (as reported in Chapter 2 of this report) provided direct confirmation for the reductive degradation of TNT followed by covalent binding to organic matter during composting of spiked soil. In general, the spectra were consistent with results from studies on the covalent binding of the individual monoamines and diamines with soil humic acid and peat. Several features of the spectra indicate covalent binding by the diamines as a significant process in the transformation of TNT into bound residues. The reduced TNT amines bind to organic matter through a broad variety of covalent bonds, including aminohydroquinone, aminoquinone, heterocyclic, and imine linkages.

The products of the interactions between TNT transformation products and humic substances are complex. The progression of covalent bonding reactions results in products exhibiting various potentials for subsequent release. However, a significant proportion of these reactions are extremely stable. Therefore, long-term stability of the products of these reactions, and, consequently, of the effectiveness of the remediation processes in which they occur, is a function of the local environment, the transformation products available for reaction, and the progression of the reactions.

Chemical Stability and Analytical Methods

The transformation of TNT to monoamino and diamino transformation products and the subsequent reactions of these products with organic material in compost and digester sludge occur in two stages. After the first few days of compost or digester sludge treatment, about 20 percent of the transformed TNT was released by hydrolysis as identifiable transformation products. After about 40 days, very little transformed TNT was released from either system. Current analytical methods of solvent extraction are effective in removing unconjugated TNT and TNT transformation products from these treatment matrices. The hydrolysis regime developed in this study is effective in removing the weakly conjugated amines that are subject to further reaction. This pool constitutes the potentially available products resulting from treatment at the stage where the analysis is conducted. When treatment takes the reaction sequence to completion, no hydrolyzable products are released. The hydrolysis method can, therefore, serve as an indicator of treatment progression and/or effectiveness. The method is not sufficiently sensitive for application to immobilization processes in soils because of the relatively small amount of organic matter in soils.

Microbial Degradation

Explosives residuals in composted TNT-contaminated soils were not degradable by microorganisms. Very few microorganisms were able to grow on the compost because it failed to provide sufficient carbon and energy. When the most degradable organic fraction, cellulose, was subjected to degradation studies

independently of the rest of the compost matrix, microorganisms were able to mineralize significant quantities of added radiolabeled TNT (up to 23 percent). Moderate mineralization activity was also observed in the fulvic acid fraction. These results demonstrate the limits of degradation potential in finished compost. Since the fractions are dispersed in the compost matrix, their degradation potential in whole compost is extremely small. Therefore, significant release of environmentally hazardous products from finished compost by microbial action is unlikely.

Toxicity

Results of Mutatox and Ames assays of TNT and the amino transformation products indicated none to moderate microbial genotoxicity. In general, the monoamino transformation products of TNT were equally or only slightly less toxic than the parent compound. However, toxicity of the compounds is best assessed in tests that take bioavailability into account. Therefore, a battery of earthworm bioassays on TNT-contaminated compost and soils were conducted. The battery included acute toxicity, chronic toxicity, avoidance behavior, metabolism and oxidative stress, immunotoxicity, and genotoxicity. The 14-day LC_{50} of TNT in soil was 353 mg kg^{-1} . Attempts to define similar toxicity for RDX and HMX were abandoned at soil concentrations of $4,000 \text{ mg kg}^{-1}$ when no earthworms died. Although HMX and RDX in soil were not acutely toxic to earthworms, their negative effect on reproduction at 400 mg kg^{-1} was severe. In earlier investigations, earthworms exhibited high chronic toxicity to finished TNT compost—only 35-percent survival after 8 days compared with 95-percent survival for controls after 21 days of exposure. Additionally, earthworms avoided the finished compost, suggesting that colonization of finished composts by earthworms is not likely. Oxidative stress effects on several enzymes and protein were observed in worms exposed to TNT-contaminated soils and composts. TNT-contaminated soils reduced immunocompetence in the worms, but was not genotoxic. However, the finished compost was highly genotoxic. The toxicity was attributed to the presence of the extractable transformation product 4ADNT, which was found at 4.55 mg kg^{-1} . However, results of studies in Chapter 3 of this report demonstrated that the residual toxicity was not due to 4ADNT. Instead, alfalfa, which was added as an amendment for composting, was toxic to earthworms. When the alfalfa was replaced with hay, the toxicity was eliminated even though chemical analysis of the hay compost was similar to that of the alfalfa compost. This is further supported by the fact that the most toxic metabolite (4ADNT) determined in the earthworm contact toxicity test had no effect on survival, growth, reproduction, or ssDNA strand breaks in the earthworms at concentrations 10-fold higher than that found in the composted material.

RDX is essentially nontoxic. The toxicities previously observed for RDX (weapons-grade) were almost entirely the result of adulteration with HMX. This is an important point for bioremediation efforts; although RDX is the more

easily degraded compound, it is less toxic than HMX, which does not compost efficiently.

The six end points for which lowest and no observed adverse effect levels (LOAELs and NOAELs) were measured (avoidance, total glutathione (21-day), postmitochondrial protein (21-day), growth, reproduction, and immunotoxicity) were sensitive indicators of explosives contamination. LOAELs and NOAELs can aid in the establishment of environmentally acceptable end points to be used in lieu of analytical chemistry data to establish cleanup and remediation levels of explosives in soils. Analytical chemistry is expensive and, in any case, not directly interpretable in terms of ecological effects. Conversely, biological end points such as growth, reproduction, and immunotoxicity are highly relevant and easily interpretable.

Summary

An understanding of the processes by which TNT becomes immobilized in remediation systems such as compost has been achieved. The chemical nature of the immobilization products and some of the factors controlling the character of these products have been defined. The long-term stability of the products of immobilization have been explored in terms of their susceptibility to chemical hydrolysis, microbial degradation potential, and toxicity. An analytical method for taking a snap-shot of the evolving products of the immobilization process has been developed. Results of this research will contribute to the optimization and regulatory acceptance of composting. Results also have important implications for other biotreatment processes including natural attenuation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)**2. REPORT DATE**

December 1999

3. REPORT TYPE AND DATES COVERED

Final report

4. TITLE AND SUBTITLE

Explosives Conjugation Products in Remediation Matrices: Final Report

5. FUNDING NUMBERS**6. AUTHOR(S)**

J. C. Pennington, K. A. Thorn, L. S. Inouye, V. A. McFarland, A. S. Jarvis,
C. H. Lutz, C. A. Hayes, B. E. Porter

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

U.S. Army Engineer Research and Development Center, Environmental Laboratory,
3909 Halls Ferry Road, Vicksburg, MS 39180-6199; U.S. Geological Survey,
National Water Quality Laboratory, P.O. Box 25046, Denver, CO 80225-0046; AScI
Corporation, 1365 Beverly Road, McLean, VA 22101

**8. PERFORMING ORGANIZATION
REPORT NUMBER**

Technical Report SERDP-99-4

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Corps of Engineers
Washington, DC 20314-1000

**10. SPONSORING/MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION/AVAILABILITY STATEMENT**

Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 words)**

Immobilization of 2,4,6-trinitrotoluene (TNT) in soils and in compost has been widely documented. Dramatic reductions in solvent-extractable TNT have been observed, while complete recoveries of radioactivity from carbon-14 labeled TNT indicated that all of the products of TNT remained in the matrix in some form. The objectives of these studies were to determine the nature and mechanism of immobilization and the long-term stability, biodegradability, and toxicity of the final products. The approach included ¹⁵N-nuclear magnetic resonance analysis of binding to humic materials, acid/base hydrolysis with subsequent identification of products, microbial degradation, and toxicity studies. Results confirm covalent bonding of the amino transformation products of TNT that is only slightly reversible, depending upon the degree of completion to which the reactions have occurred. Products were resistant to microbial degradation and relatively stable against hydrolysis. Toxicity was related to bioavailability, which depended upon the degree of completion of the immobilization reactions. These results have important implications for fate of TNT in remediation systems and in the environment.

14. SUBJECT TERMS

Bioavailability	Explosives	TNT
Covalent bonding	Humification	
Earthworm bioassays	Immobilization	

15. NUMBER OF PAGES

50

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

UNCLASSIFIED

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

UNCLASSIFIED

**19. SECURITY CLASSIFICATION
OF ABSTRACT****20. LIMITATION OF ABSTRACT**